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Licenciada em Biologia
Mestre em Genética Molecular e Biomedicina

Assessing antibiotic resistance in Gram negative bacteria from animals and the wider environment

Dissertação para obtenção do Grau de Doutor em Biologia

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RESUMO

A emergência da resistência aos antibióticos na medicina humana lançou o apelo para uma compreensão ampla e concertada deste evento. A presente dissertação teve como principal objectivo investigar os determinantes de resistência aos antibióticos transferíveis entre bactérias de Gram negativo, destacando o contributo de elementos genéticos móveis para a sua disseminação. Os estudos de susceptibilidade e epidemiologia molecular, realizados em colecções de isolados bacterianos demonstraram a predominância de *Escherichia coli* com multiresistência aos antibióticos, quer em amostras provenientes de animais, quer de solos e vegetais. Tal como *E. coli*, outras bactérias de Gram negativo estavam associadas a genes de resistência com importância clínica [tais como $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{CMY-2}}$, $bla_{\text{GES-11}}$, *qnrS1*, *aac(6')-Ib-cr*, *strAB*, *tet, drfA, aadA*, *mcr-1*], por sua vez, incorporados em suportes genéticos mobilizáveis. De facto, a diversidade de elementos genéticos móveis (por exemplo, plasmídeos do tipo IncI1 e IncF, sequências de inserção IS*Ecp1*, transposões Tn*402* e Tn*7* e integrões de classes 1, 2 e 3) detectados na proximidade desses genes sugeriram o seu envolvimento, não só na dispersão interespécies dos determinantes de resistência, como também na sua movimentação no interior da célula bacteriana. A investigação do genoma de dois isolados de *Enterobacteriaceae* e do proteoma de um terceiro, sublinhou o potencial das abordagens *ómicas* no estudo da resistência aos antibióticos. A principal conclusão emanada desta análise revelou que a resistência aos antibióticos, não só está directamente associada à patogenicidade, como está também relacionada com processos metabólicos centrais. Os resultados obtidos com a presente dissertação de doutoramento amplificaram o conhecimento científico existente relativo à distribuição de genes móveis de resistência aos antibióticos em animais, no ambiente e, em última instância, na cadeia alimentar. Se os resultados alcançados elevaram a preocupação existente acerca da emergência da resistência aos antibióticos que sirvam também como motivação para abordar este problema de uma forma global.

Palavras-chave: resistência aos antibióticos, elementos genéticos móveis, disseminação, agricultura, ambiente, animais.

ABSTRACT

The alarming increase in the levels of antibiotic resistant bacteria in clinical practice launched the call for a broader understanding of this event. This Ph.D. thesis aimed to unravel the main mobile antibiotic resistance determinants circulating in Gram negative bacteria from non-human sources, showing the contribution of mobile genetic elements for the overall process. The susceptibility and molecular epidemiological studies performed on different collections of bacterial isolates showed the predominance of multidrug resistant *Escherichia coli* in animals of different origins, soil and vegetables. *E. coli* and other species of Gram negative bacteria were related with carriage of diverse antibiotic resistance genes [e.g. $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{CMY-2}}$, $bla_{\text{GES-11}}$, *qnrS1*, *aac(6')-Ib-cr*, *strAB*, *tet, drfA, aadA*, *mcr-1*] that were associated with a transferable genetic support. Indeed, an assortment of mobile genetic elements (e.g. IncI1 and IncF plasmids, IS*Ecp1* insertion sequences, Tn*402* and Tn*7* transposons and class 1, 2 and 3 integrons) was detected in the genetic proximity of those antibiotic resistance genes, suggesting their profound involvement, not only in interspecies dispersion, but also in the movement of the genes within the cell. Specific genomic investigation of two *Enterobactericeae* isolates and the proteomic study of a third, underscored the potential of massive *omic* approaches to study antibiotic resistance as a global process. One of the key findings released from these studies is that antibiotic resistance is not only linked to virulence and pathogenicity, but can also be connected to core bacterial metabolic processes. The results obtained throughout this thesis extend our knowledge on the distribution of mobile antibiotic resistance genes in animals, environment and, ultimately, in the food chain. If the gathered results increased our concerns towards the current distribution of antibiotic resistant bacteria, they can also be an encouragement to address this problem at a global scale.

Key-words: antibiotic resistance, mobile genetic elements, dissemination, agriculture, environment, animals.

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LIST OF ABBREVIATIONS

Aac(6')-Ib-cr, Aminoglycoside Acetyltransferase variant cr

AAC, Aminoglycoside Acetyltransferase

AAD, Aminoglycoside Adenylyltransferase

ACT, AmpC Type

ANT, Aminoglycoside Adenylyltransferase

APH, Aminoglycoside Phosphotransferase

attC, Attachment site of the integrons cassette

attI, Attachment site of the integron

bla, β-Lactamase coding gene

CAT, Chloramphenicol Acetyltransferases

CC, Clonal Complex

CDC, Centers for Disease Control and Prevention

CECA-ICETA, Centro de Estudos de Ciência Animal - Instituto de Ciências e Tecnologias Agrárias e Agro-Alimentares

CHDL, Carbapenem-Hydrolyzing Class D β-Lactamase

CI, Confidence Interval

CmlA, Chloramphenicol resistance determinant A

CMY, Active on Cephamycins

CTX-M, Active on Cefotaxime, first isolated in Munich

DfrA, Dihydrofolate reductase

DHA, Discovered at Dhahran, Saudi Arabia

DNA , Deoxyribonucleic acid

e.g. exempli gratia, for example

ECOFF, Epidemiological Cut-Off values

ECDC, European Centre for Disease Prevention and Control

EFSA, European Food Safety Authority

EMA, European Medicines Agency

ESBL, Extended-Spectrum β-Lactamase

et al., et alia, and other people

EUCAST, European Committee on Antimicrobial Susceptibility Testing

FloR, Florfenicol Resistance

GES, Guiana Extended-Spectrum

HGT, Horizontal Gene Transfer

ICE, Integrative and Conjugative Element

IMP, Active on Iminipem

Inc, Incompatibility group

INIAV, Instituto Nacional de Investigação Agrária e Veterinária

INSA, Instituto Nacional de Saúde Doutor Ricardo Jorge

IntI, Integron Integrase

IS, Insertion Sequence

ISCR, Insertion Sequence Common Region

KPC, *Klebsiella pneumoniae* Carbapenemase

LC-MS, Liquid Chromatography–Mass Spectrometry

MBL, Metallo-β-Lactamase

MDR, Multidrug Resistance

MGE, Mobile Genetic Element

MIC, Minimum Inhibitory Concentration

MLST, Multilocus Sequence Typing

MS, Mass-Spectrometry

NDM, New Delhi Metallo-β-lactamase

NGS, Next Generation Sequencing

NMC, Not Metalloenzyme Carbapenemase

NRL-AMR/HAI, National Reference Laboratory of Antibiotic Resistances and Healthcare Associated Infections

OMP, Outer Membrane Protein

OMV, Outer Membrane Vesicles

OqxAB, Olaquindox Resistance

OR, Odds Ratio

Otr, Oxytetracycline Resistance

OXA, Active on Oxacillin

PBP, Penicillin Binding Protein

PBRT, PCR-Based Replicon Typing

Pc, Promoter of Cassete

P*intI*, Promoter of Integrase Integron

PCR, Polymerase Chain Reaction

PER, *Pseudomonas* Extended Resistant

PFGE, Pulsed-Field Gel Electrophoresis

PGAAP, NCBI Prokaryotic Genome Automatic Annotation Pipeline

PMAβ, Plasmid-Mediated AmpC β-lactamase

pMLST, Plasmid Multilocus Sequence Typing

PMQR, Plasmid-Mediated Quinolone Resistance

QepA, Quinolone Efflux Pump A

Qnr, Quinolone Resistance

QRDR, Quinolone-Resistance Determining Region

RAST, Rapid Annotation using Subsystem Technology

RBPs, Ribossomal Binding Proteins

RPPs, Ribossomal Protection Proteins

SHV, Sulphydryl Reagent Variable

SME, *Serratia marcescens* Enzyme

ST, Sequence Type

Sul, Sulfonamide resistance

TEM, Named after patient Temoniera

Tet, Tetracycline resistance

UPGMA, Unweighted Pair Group Method with Arithmetic Mean

VEB, Vietnam Extended-Spectrum β-lactamase

VIM, Verona Integron-encoded Metallo-β-lactamase

WGS, Whole Genome Sequencing

WHO, World Health Organization

THESIS OUTLINE

Infections caused by antibiotic resistant bacteria have ascended in the last decades into one of the most significant worldwide causes of death. In Europe, real efforts have been implemented in order to control this development. Many working groups, networks and guidelines have been created with the purpose of establishing policies and goals, not only for the clinical management of antibiotic resistance, but also for the detection of relevant antibiotic resistance mechanisms produced by pathogenic agents. However, the containment of such pathogens cannot be limited to clinical microbiology diagnostics and research. In the end, the environment is involved in the dynamics of antibiotic resistance, by originally supplying environmental antibiotic resistance genes and by becoming contaminated with biopollutants, acting as a ground for new recruitment. Thus, the study of the environmental pools of antibiotic resistance and the mechanisms involved in their emergence and mobilization are crucial to keep up with this phenomenon and to develop solutions intended to minimize their impact.

The main body of this Ph.D. dissertation is based on ten manuscripts that are presented as individual chapters (3 to 12). Five of them have already been published and the remaining research papers are submitted or accepted for publication in international peer reviewed journals. Each research paper-based chapter is preceded by a title page describing the reference of the publication and the contributions of each author, as well as a specialized introduction, material and methods, results and discussion concerning the scope of the manuscript. It should be noted that the order in which they are included in the thesis does not reflect a chronological arrangement. Besides these manuscript-based chapters, the present Ph.D. thesis includes an initial overview on antibiotic resistance (Chapter 1, General introduction), followed by the objectives (Chapter 2, Aim of the thesis), and a global discussion that correlates all the gathered results (Chapter 13, General discussion and conclusions).

Considering the different article types and layouts adopted by the journals in which the manuscripts were published or submitted, the chapters (3 to 12) were formatted in a single style, with all references listed together in the "References" section. Finally, annexes relative to each chapter were also compiled in a last section of this Ph.D. dissertation in a section denominated "Supplemental material". For the sake of simplicity, a link to an online document is provided for some extensive supplementary data. Numbering of figures and tables is presented according with the number of the chapter.

The specific contents of each chapter are enclosed in this Ph.D. thesis, as follows:

Chapter 1 consists of a general introduction that intents to supply the reader with the state of the art in antibiotic resistance, mainly focused on Gram negative bacteria. Thus, it is firstly given a global overview of the impact of antibiotic resistance, followed by insights into the origins and evolution of this event and the mechanisms of action and resistance towards the most relevant antibiotic classes. In the end, the contributions of the *omic* era to the study of antibiotic resistance are depicted. The issues focused on this chapter were put into context to emphasize the relevance of the presented studies.

Chapter 2 includes the scope, and description of the main and specific objectives of this Ph.D. work.

Chapters 3 to 6 include the study of *Escherichia coli* and *Salmonella* spp. isolates recovered from animals of different origins: companion, zoo and/or food-producing animals.

In **Chapter 3** we have evaluated the molecular basis of transferable quinolone resistance in *E. coli* and *Salmonella* spp. recovered from food-producing animals and food products collected in Portugal.

Chapter 4 comprises the investigation of a new class 2 integron In*2-4* detected among IncI1-positive *E. coli* isolates carrying extended-spectrum β-lactamases- and plasmid-mediated β-lactamases-encoding genes from food animals, also recovered in Portugal.

In **Chapter 5,** plasmid-mediated quinolone resistance determinants QnrS1 and Aac(6')-Ib-cr, produced by *E. coli* isolates, were investigated. These isolates were part of a collection of *Salmonella enterica* and *E. coli* recovered from companion, zoo and food-producing animals that were also evaluated regarding antibiotic susceptibility.

In **Chapter 6** we evaluated the zoonotic potential of an *E. coli* isolate recovered from a captive bottlenose dolphin by assessing the genetic relatedness between this isolate and a group of human clinical isolates with similar phenotypic and genotypic characteristics.

In Chapters 7 to 9, our investigation was directed to multiple species of antibiotic resistant Gram negative bacteria associated with different agricultural practices, and isolated from crop soil and vegetables recovered from retail stores.

Chapter 7 includes the study of the culturable antibiotic resistant Gram negative population contained in different agricultural soils. Subsequent evaluation of antibiotic

susceptibility and further molecular characterization showed the presence of IncI1 plasmids carrying various β-lactamase-encoding genes in *E. coli* from intensive agrosystems.

In **Chapter 8**, the presence and architecture of twelve class 1, 2 and 3 integrons was evaluated in Gram negative bacteria recovered from organic and conventionally produced fruits and vegetables, after assessment of antibiotic susceptibility of the bacterial collection and molecular investigation of the integron-producing isolates.

Chapter 9 comprises an in-depth short report of an *E. coli* isolate carrying the recently discovered plasmid-mediated colistin resistance gene (*mcr-1*), which was recovered from a non-imported vegetable acquired at a large retail store.

Chapters 10 to 12 include a thorough study of three specific isolates recovered during this Ph.D. thesis using massive genomic and proteomic approaches.

In **Chapter 10** we used a genomic approach to investigate the molecular traits related with pathogenicity, multidrug resistance and a new plasmid in an avian antibiotic resistant *qnrD1*-harboring *Morganella morganii*.

Chapter 11 consists on the report of the draft genome sequence of a pathogenic O86:H25 sequence type 57 *E. coli* strain isolated from poultry and carrying 12 acquired antibiotic resistance genes.

In **Chapter 12** we used a quantitative proteome approach to analyze an antibiotic resistant *E. coli,* previously recovered from soil, when exposed to a high dosage of tetracycline. This study revealed that in *E. coli* multiple metabolic and peptidoglycan cytoplasmic processes were affected by antibiotic-induced stress.

Finally, **Chapter 13** includes a global and conclusive overview of the themes addressed throughout this Ph.D. dissertation, underscoring the main results and conclusions accomplished.

Chapters 3 to 12 may be read separately and transcribe the contents of the following publications:

- Chapter 3: Jones-Dias, D., Manageiro, V., Francisco, A.P., Martins, A.P., Domingues, G., Louro, D., Ferreira, E., Caniça, M. 2013. *Veterinary Microbiology* 167:523-31.

- Chapter 4: Jones-Dias, D., Manageiro, V., Martins, A.P., Ferreira, E., Caniça, M. 2016. *Foodborne Pathogens and Disease* 3: 36-39.
- Chapter 5: Jones-Dias, D., Manageiro, V., Graça, R., Sampaio, D.A., Albuquerque, T., Themudo, P., Vieira, L., Ferreira, E., Clemente, L., Caniça, M. 2016. *Frontiers in Microbiology* (accepted).
- Chapter 6: Manageiro, V., Clemente, L., Jones-Dias, D., Albuquerque, T., Ferreira, E., Caniça, M. 2015. *Emerging Infectious Diseases* 21: 2249-2251.
- Chapter 7: Jones-Dias, D., Manageiro, V., Caniça, M. 2016. *Environmental Microbiology* 21: 2249-2251.
- Chapter 8: Jones-Dias, D., Manageiro, V., Ferreira, E., Barreiro, P., Vieira, L., Moura, I. B., Caniça, M. 2016. *Environmental Microbiology* (submitted).
- Chapter 9: Jones-Dias, D., Manageiro, V., Vieira, L., Ferreira, E., Caniça, M. 2016. *Journal of Antimicrobial Chemotherapy* (submitted).
- Chapter 10: Jones-Dias, D., Clemente, L., Moura, I.B., Sampaio, D.A., Vieira, L., Manageiro, V., Caniça, M. 2016. *Frontiers in Microbiology* (submitted).
- Chapter 11: Jones-Dias, D., Manageiro, V., Sampaio, D.A., Vieira, L., Caniça, M. 2015. *Genome Announcements*. 3: e01107-15.
- Chapter 12: Jones-Dias, D., Carvalho, A. S., Moura, I., Igrejas, G., Caniça, M., Matthiesen, R. 2016. *Frontiers in Microbiology* (submitted).

To my parents and to Carlos.

Chapter 1.

General introduction

1.1. Clinical impact of antibiotic resistance

"The time has come to close the book on infectious diseases"

William Stewart, 1967

Antibiotics[1] constitute the most successful drugs meant to improve human health. Their introduction as a treatment of infectious diseases allowed a remarkable increase in life expectancies all around the world, to the point where infections were considered a closed case (Upshur, 2008). Beyond the treatment of nosocomial community acquired infections, the use of antibiotics allowed the implementation of innovative clinical procedures such as immunosuppression associated with transplants or cancer therapy, extensive surgery, catheterization, and assisted life support, among others. These antimicrobial agents have not only revolutionized medicine but have also saved countless lives throughout the years (Upshur, 2008; Martínez and Baquero, 2014).

During the golden age of antibiotic development (somewhere between the years of 1940 and 1960), approximately 150 types of antibiotics were developed (Figure 1.1). However, in the 1970s, a decreasing interest and ability of the pharmaceutical industry to create new antibiotics resulted in a 40 year period during which no new classes of antibiotics were released and commercialized (Dantas and Sommer, 2014). Since the emergence of the first cases of antibiotic resistance, which comprised the resistance to penicillins in the mid 40's, antibiotic resistance has increased, rapidly exceeding the rate of development of new antibiotics (Figure 1.1) (Abraham and Chain, 1988).

Today, infectious diseases constitute one of the major causes of illness worldwide, and antibiotic resistance has emerged as one of the major priorities of human medicine (Giske et al., 2008). Regrettably, instead of bacteria resistant to single classes of antibiotics, pathogens now frequently harbor multidrug resistance, which constitutes a serious therapeutic challenge that often cannot be overcome. Lately, the efforts to combat multidrug resistant bacteria have been mainly focused on Gram positive microorganisms, which led to the implementation of essential infection control protocols and to the

[1] In this thesis, and as initially proposed by Selman Waksman, the generic term "antibiotic" will be used to denote any class of molecule that kills bacteria (bactericidal) or inhibits their growth (bacteriostatic) through specific interactions with a bacterial target (Waksman et al., 1973).

development of novel antibiotics, such as daptomycin or tigecycline. However, no new antibiotics have been developed specifically for Gram negative bacteria, leading to a situation where the impact of multidrug resistant Gram negative bacilli is devastating (Giske et al., 2008). These bacteria include certain strains of *Escherichia coli* and *Klebsiella pneumoniae* that are resistant to all major classes of antibiotics, including carbapenems, which have long been used as a last resort drug for the treatment of many complicated infections caused by Gram negative bacteria (Munoz-Price et al. 2013; Dantas and Sommer, 2014). In fact, carbapenem resistance among common *Enterobacteriaceae* has greatly increased over the past decade (Hawkey, 2015). Multidrug resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* are also among the pathogenic agents that cause the highest burdens, being associated with high mortality rates (Laxminarayan et al., 2013).

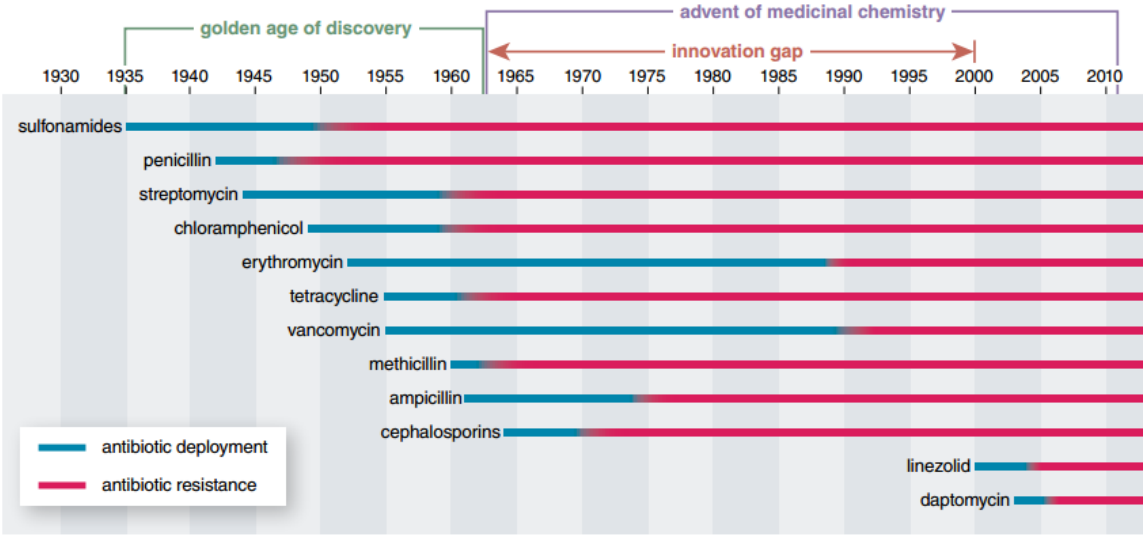

Figure 1.1. Deployment of new antibiotics has been accompanied by the development of bacterial resistance (Dantas and Sommer, 2014).

Undeniably, antibiotic resistance not only has a significant human impact, but it also has high economic consequences. According to a recent report from the Centers for Disease Control and Prevention (CDC), the treatment of antibiotic resistant infections adds $35 billion in healthcare costs, and 8 million hospital days per year in the United States of America (CDC, 2014). A late antibiotic resistant *Salmonella* spp. outbreak, due to contaminated chicken meat, was linked to near 300 cases distributed by 18 states, sickening both infants and the elderly alike. The Infectious Disease Society of America estimates that 70% of bacteria causing hospital acquired infections in the United States are now resistant to one or more antibiotics. Globally, at least 23,000 Americans die each

year from infections (CDC, 2014; Dantas and Sommer, 2014). The European data is far from being more optimistic. In 2009, the European Centre for Disease Prevention and Control (ECDC) and the European Medicines Agency (EMA) estimated that each year 25,000 Europeans die as a direct consequence of infections caused by multidrug resistant microorganisms, with an estimated economic impact of €1.5 billion (EMA, 2009).

According to the previously mentioned CDC's report, there are four core actions that will help fighting antibiotic resistant bacteria: 1) preventing infections and the spread of resistance, 2) tracking antibiotic resistant microorganisms, 3) improving the use of antibiotics, and 4) promoting both the deployment of new antibiotics and the development of new rapid diagnostic tests for antibiotic resistant bacteria (CDC, 2014).

Despite all efforts against antibiotic resistance, bacteria will inevitably find new ways to resist, which is why hostile action is needed to keep new resistance from emerging and to prevent the resistance that already exists from spreading (Dantas and Sommer, 2014). Learning the causes for the evolution of antibiotic resistance with the aim of preventing its emergence is an even more urgent necessity than it was before.

1.2. On the origins and evolution of antibiotic resistance

"It is not the strongest species that survives, nor the most intelligent but the one most responsive to change"

Charles Darwin, 1809

1.2.1. Unraveling the antibiotic resistome

The main reason for the calamity of antibiotic resistance is also one of the most remarkable aspects of modern evolutionary processes: the rapid ability of susceptible microorganisms to adapt, surviving the exposure to antibiotics (Martínez et al., 2009).

The global source of resistance genes is starting to become understood. Scientific

evidences support the existence of a large bacterial resistome - the collection of all resistance genes and their precursors in both pathogenic and nonpathogenic bacteria (D'Costa et al., 2006). It is known that resistance mechanisms identified in pathogenic bacteria did not emerge in the clinical setting but instead in environmental bacterial populations, before the use of antibiotics by humans (Martínez, 2009; Martínez and Baquero, 2014; Martínez et al., 2015). Antibiotic resistance determinants were once encoded by genes that were originally selected for the transport of small molecules and signal trafficking, among other metabolic functions (Olivares et al., 2013). It is estimated that the antibiotic resistance genes now known may have been acquired directly from antibiotic-producing microorganisms that harboured them as a self protection mechanism. Subsequently, they evolved and spread naturally in response to the selection pressure caused by exposure to higher and external concentrations of antibiotics (Dantas and Sommer, 2012; Berendonk et al., 2015). Many examples of this situation can be enumerated: antibiotic-inactivating enzymes such as β-lactamases or aminoglycoside acetyl transferases, whose main function is the modification of the peptidoglycan layer; multidrug efflux pumps that can serve to extrude intercellular signals or toxic compounds; or other hypothetical proteins that are unlikely to have a primary role on resistance given that they provide resistance to synthetic quinolones, which were not present in natural ecosystems before their development, such as resistance to these antibiotics provided by the Qnr proteins (Poirel et al., 2012; Olivares et al., 2013).

Overall, two main findings are rising from the research of the natural resistome. First, the environmental microbiota harbors a much superior amount of resistance genes than those that were recruited by pathogenic agents (Martínez, 2012). In fact, different environments contain different resistance genes; this means that we are still far from having a reliable estimation on the quantity of potential resistance genes present in natural ecosystems. Second, the genes present in mobile genetic elements (MGE) in human pathogens can be found virtually everywhere, including pristine ecosystems and wild animals, not supposed to be in contact with antibiotic residues. However, one can argue that it is virtually impossible to stay clear of antibiotic contamination, especially when in the top of the food chain (Marinho et al., 2014; Sousa et al., 2014). This indicates that pollution with antibiotic resistance genes is widely spread and that resistance genes can persist even in the absence of a positive selection pressure (Martínez, 2012; Olivares et al., 2013; Perry and Wright, 2013).

1.2.2. Emergence and dissemination of antibiotic resistance genes

Despite the existence of antibiotic-producing bacteria and precursors of antibiotic resistance genes in the environment, their transboundary emergence and spread is a multifactorial process that involves genetic mechanisms and external environmental factors (Olivares et al., 2013).

Resistance can emerge by mutation within populations of bacteria and be retained by subsequent selection and further vertical transmission, or by the horizontal acquisition of resistance determinants from other microorganisms (Figure 1.2).

In vertical transmission, a bacterium accumulates mutations in its genome during replication; some of those changes provide them with advantages such as the ability to resist antibiotics, which are passed on to subsequent generations (Dantas and Sommer, 2014). This is a fundamental evolutionary process by which a bacterial cell can accumulate alterations in its DNA, in such a way that the resulting offspring differs genetically from their bacterial ancestors. When bacterial mutants are exposed to antibiotics, those carrying genes that guarantee antibiotic resistance will increase its prevalence (Gillings and Stokes, 2012; Dantas and Sommer, 2014).

Genes that have evolved to confer antibiotic resistance can also be transferred by horizontal gene transfer (HGT) (also denominated lateral gene transfer), in a process where resistant genes are exchanged between different microorganisms (Stokes and Gillings, 2011); this can occur through three mechanisms: transformation, transduction and conjugation (Figure 1.2). Transformation occurs when bacteria incorporate naked DNA, such as antibiotic resistance genes, released upon the lysis of dead bacterial cells and integrate them into their own genomes. Transduction takes place when resistance genes are transferred between bacteria by bacteriophages and can be integrated into the chromosome of the recipient cell. Conjugation occurs when genes are intentionally transferred between prokaryotes through a process involving conjugative *pili* that results in the exchange of DNA (Furuya and Lowy, 2006).

The accumulation of studies focusing on the horizontal gene transfer of clinically important antibiotic resistance genes suggests that this process may be the dominant force behind growing antibiotic resistance and a major threat to the confinement of new

resistance mechanisms within specific bacteria or restricted settings (Leverstein-van Hall et al., 2011; Chen et al., 2014a; de Been et al., 2014). In fact, horizontal gene transfer brings a major contribution to the evolution of the prokaryotic genome and dissemination of the acquired determinants (Norman et al., 2009). The acquisition of antibiotic resistance genes by horizontal gene transfer can be mediated by a variety of mobile genetic elements, such as plasmids, phages or transposons, among many others. To better understand the dynamic of genetic mobility associated to antibiotic resistance it is necessary to look closer at some of the elements and processes that manage these rearrangements within the global resistome (Stokes and Gillings, 2011).

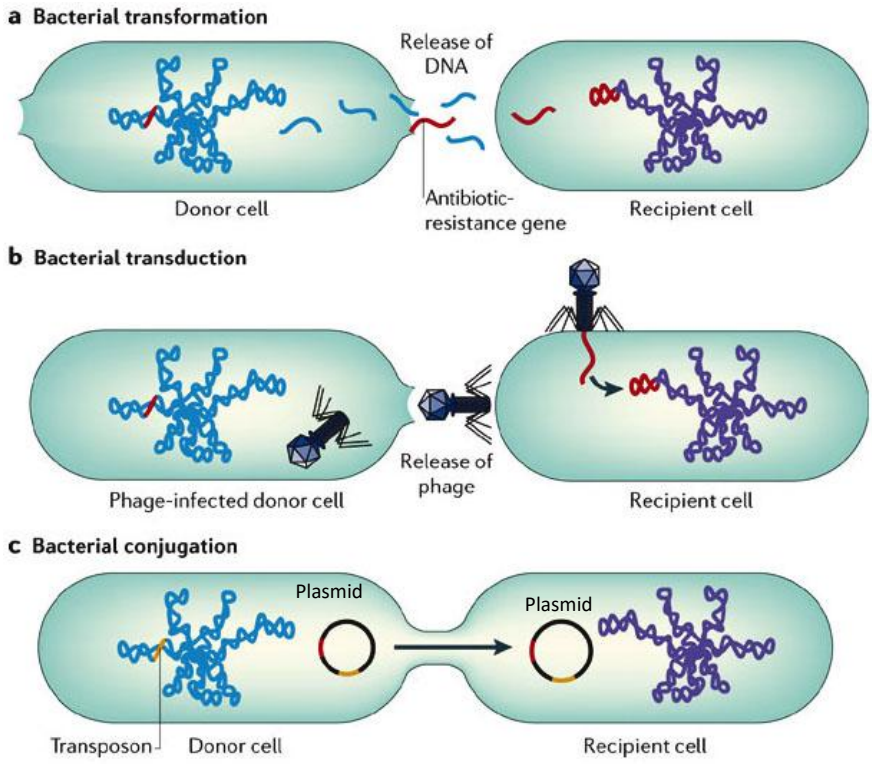

Figure 1.2. Horizontal gene transfer driven by bacterial transformation (a), bacterial transduction (b) and bacterial conjugation (c) (Furuya and Lowy, 2006).

The mobilization of any gene relies on a set of molecular mechanisms that manipulate and translocate specific DNA regions. This is achieved by a set of enzymes which include recombinases that mediate homologous recombination, transposases, which catalyze the movement and insertion of transposons, integrases that enable insertion of elements such as gene cassettes into integrons by site-specific recombination, and resolvases, which are DNA endonucleases involved in genetic recombination. These enzymes are enclosed in mobile elements that are able to promote gene capture and guarantee their expression. In

fact, they all play an essential part in horizontal gene transfer and in the mobilization and recruitment of antibiotic resistance genes (Norman et al., 2009).

Mobile genetic elements fall into two main categories: elements that can move from one bacterial cell to another through a replication-mediated mechanism and structures that can move from one genetic location to another within the same cell. The first include resistance plasmids and integrative conjugative elements, the second require a recombination mechanism that may or may not comprise a form of replication, such as some transposons, gene cassettes and Insertion Sequence Common Regions (ISCRs) (Bennett, 2008; Norman et al., 2009; Toleman and Walsh, 2011). Hence, at least three different recombination systems can act simultaneously to accumulate and re-assort resistance genes in bacterial plasmids. The molecular properties of the main groups of mobile genetic elements associated with antibiotic resistance are further depicted in Table 1.1.

Moreover, diverse combinations of genetic elements may arise: gene cassettes can be inserted into integrons that comprise part of transposons, which are, in turn, carried on plasmids (Bennett, 2008). Understanding the origins of antibiotic resistance-associated mobile elements is complex due to the fact that they often form mosaics, built up from different genes and elements, each carrying different evolutionary histories (Bennett, 2008). The complexity of such genetic platforms allows them to interact with other genetic elements, promoting exchanges that generate more diversity (Stokes and Gillings, 2011).

Plasmids are particularly efficient since they can harbor several resistance determinants, as well as other mobile genetic elements within (Bennett, 2008). For instance, pKPp048 plasmid (Figure 1.3) not only carries resistance to several antibiotics such as carbapenems, cephalosporins, fluoroquinolones, and aminoglycosides, but also other 163 putative genes, 108 of which were assigned functions of replication, stable inheritance, antibiotic resistance, mobile element, conjugal transfer, and restriction modification systems, highlighting the phylogenetic mosaicism and plasticity that plasmids might display (Jiang et al., 2010) (Figure 1.3).

In addition, some of the described mobile genetic elements are extraordinarily abundant. For instance, transposases are ubiquitous and one of the most abundant genes in nature (Aziz et al., 2010). In fact, a recent study by Forsberg and colleagues (Forsberg et al., 2014) suggested that it is the existence of mobile genetic elements, and thus the mobilization process, rather than the supply of antibiotic resistance genes, which limits their transfer among different groups of bacteria.

Table 1.1. Main groups of mobile genetic elements and their molecular features.

MGE	Molecular features
Plasmids	Plasmids are transferable genetic elements capable of autonomous replication. They can be either self-transmissible (conjugative) or mobilizable (non-self-transmissible). While the first group encodes a complete conjugative DNA transfer apparatus (*tra* functions), the second group usually bears only the functions required for initiation of its own transfer DNA replication (*mob* genes)(Bennett, 2008; Mata et al., 2012).
Insertion sequences	Insertion sequences are the simplest transposable elements; by definition, they carry only the genetic information necessary for insertion functions and no accessory genes (for example, drug resistance); flanked by short terminal inverted-repeat sequences of 10-40bp, they are able to insert at multiple sites in target DNA (Bennett, 2008).
ISCRs	Insertion Sequence Common Regions (ISCRs) are insertion sequences that have similarities to the IS*91* family in both structure and function. These elements are known to move by a process called rolling-circle replication, which drives the concomitant movement of sequences located upstream of their transposase genes (Bennett, 2008).
Transposons	Transposons are genetic elements that physically transpose from one genetic position to another, within the chromosome or plasmid in which they reside. Some transposons carry one or more antibiotic resistance genes in their central regions and contain insertion sequences with short inverted repeats at their termini (Bennett, 2008).
ICEs	Integrative conjugative elements (ICEs), once called conjugative transposons, are integrative DNA elements that excise themselves to form a covalently closed circular intermediate. This circular intermediate can either reintegrate in the same cell or be transferred by conjugation to a recipient, integrating that genome (Wozniak and Waldor, 2010).
Integrons and gene cassettes	Integrons are DNA elements with the ability to capture genes, by site-specific recombination. Integrons have an integrase gene (*IntI*) to mediate excision and site-specific integration of gene cassettes, a recombination site (*attI*), and a promoter (*Pc*) which ensures expression of the operon (Cambray et al., 2010). Although integrons themselves are normally not mobile they incorporate mobile gene cassettes and are often associated with transposons such as Tn*402* and Tn*7*.
Phages	Phages are obligate intracellular parasites that multiply inside bacteria by making use of some or all of the host biosynthetic machinery. They are also able to mediate the transfer of resistance genes (Reardon, 2014).

Bacteria can also accumulate advantageous genes, such as those associated with virulence, and genes encoding resistance to heavy metals, disinfectants or detergents within the same mobile genetic units, allowing resistance to specific antibiotics to be maintained

through co-selection processes. In theory, with the involvement of mobile genetic elements, any gene in any microorganism, anywhere in the world, might be mobilized and spread (Carattoli, 2013).

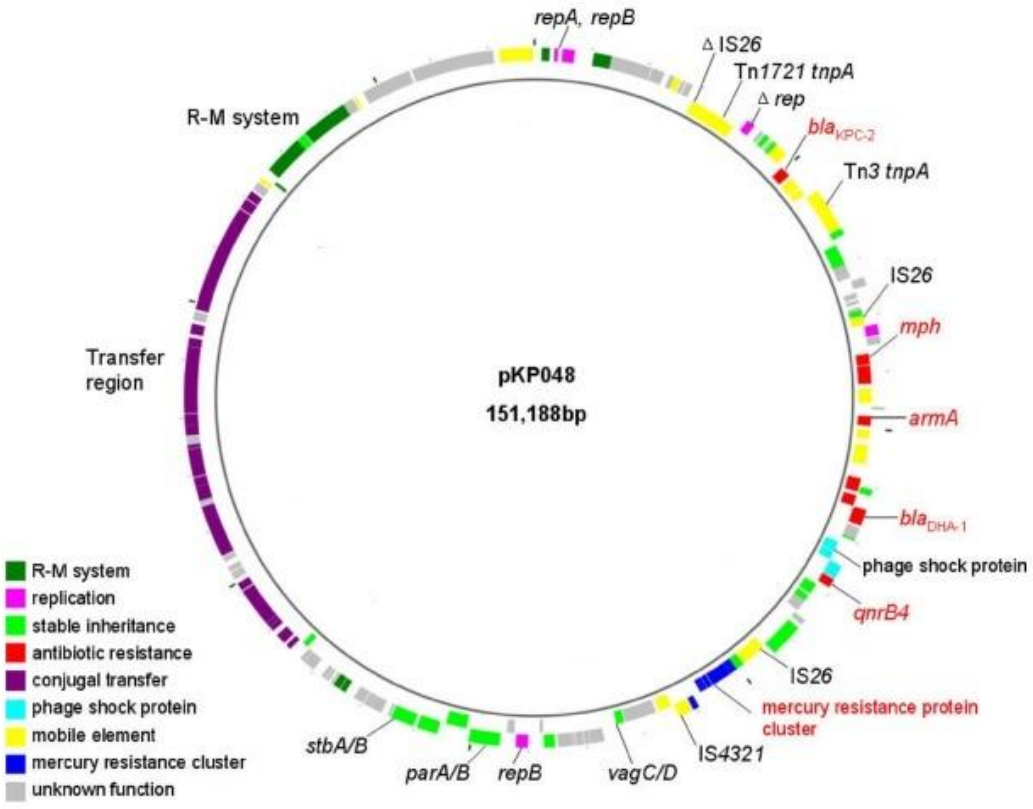

Figure 1.3. Circular map of plasmid pKP048 encoding several antibiotic resistance genes (red) and mobile genetic elements (yellow) (Jiang et al., 2010).

Thus, this dilemma can only be managed by understanding the problem from a broad ecological and evolutionary perspective, and mobile genetic elements can often be used as a genetic marker to track the dissemination of antibiotic resistance (Stokes and Gillings, 2011; Gillings and Stokes, 2012).

1.2.3. The current epidemiology of antibiotic resistance

Growing scientific evidence indicates that essentially all microbial environments contain specific antibiotic resistomes. Moreover, countless natural and human-driven activities

influence their selection and exchange between environments, resulting in a complex web of interactions that connects the various resistomes (Cox and Wright, 2013; Olivares et al., 2013).

Since industrialization, millions of tons of antibiotics have been released into the environment. For instance, 71% of total Danish antibiotic consumption in 2010 was for animal production, while in the United States antibiotics are used four times as often in the food industry as in human medicine (Wegener, 2012).

Indeed, after the use of antibiotics in human medicine, these drugs and respective metabolites are frequently discarded to the sewage system where they can become degraded, associated with sewage sludge or released to rivers, depending on their chemical characteristics (Furuya and Lowy, 2006; Caniça et al., 2015). The molecules can then enter agricultural systems either when the sludge is used as fertilizer or directly through irrigation with wastewaters and surface waters. Veterinary pharmaceuticals and their metabolites can be released into the environment either directly, from use in aquaculture and the treatment of animals on the field, or indirectly during the application of manure from intensive livestock facilities to the soil. The compounds that are released to the soil system can subsequently be transported to surface water or groundwater and re-cycled within the environment, subjecting the environmental bacteria to a persistent exposition to exogenous drug residues and antibiotic resistant bacteria. In the end, these nutrient-enriched habitats become hotspots for horizontal gene transfer (Furuya and Lowy, 2006; Cantas et al., 2013; Berendonk et al., 2015; Caniça et al., 2015).

Overall, many anthropogenic activities, including animal production, agriculture, aquaculture, household, and industrial waste disposal constitute permanent reserves of antibiotic residues, antibiotic resistant strains and antibiotic resistance genes (Dantas and Sommer, 2014; Woodford et al., 2014) (Figure 1.4). Moreover, multiple routes of interconnections between people, animals, and the environment, facilitate the transmission of antibiotic resistant bacteria between species, enhancing the evolution and spread of resistance (Figure 1.4). Soil microbes likely represent the major evolutionary reservoir of resistance, and the soil resistome is easily the largest and most diverse of any environment. In addition to those found in soil, bacteria from agriculture (from both animal and vegetable production) and aquaculture are believed to considerably contribute to this trade of antibiotic resistance determinants (Dantas and Sommer, 2014) (Figure 1.4). Agriculture and aquaculture-associated antibiotic resistant microorganisms most likely act as intermediates between the human pathogens and bacteria living in other

environments, such as those associated to livestock, soil, sea, and freshwater (Veldman et al., 2011).

Many studies have shown the potential of antibiotic resistant strains and antibiotic resistance genes to spread directly or indirectly between different settings (Heuer et al., 2011; Hartmann et al., 2012; Subbiah et al., 2012).

Genetic and genomic studies showed the presence of the same genes, plasmids and strains among Dutch patients, poultry and retail chicken meat (Leverstein-van Hall et al., 2011). In Portugal, a study described the existence of cross-contamination with resistant *Salmonella* spp. in swine abattoirs where the same bacteria were detected in carcasses, meat and meat handlers (Gomes-Neves et al., 2014; Couto et al., 2015).

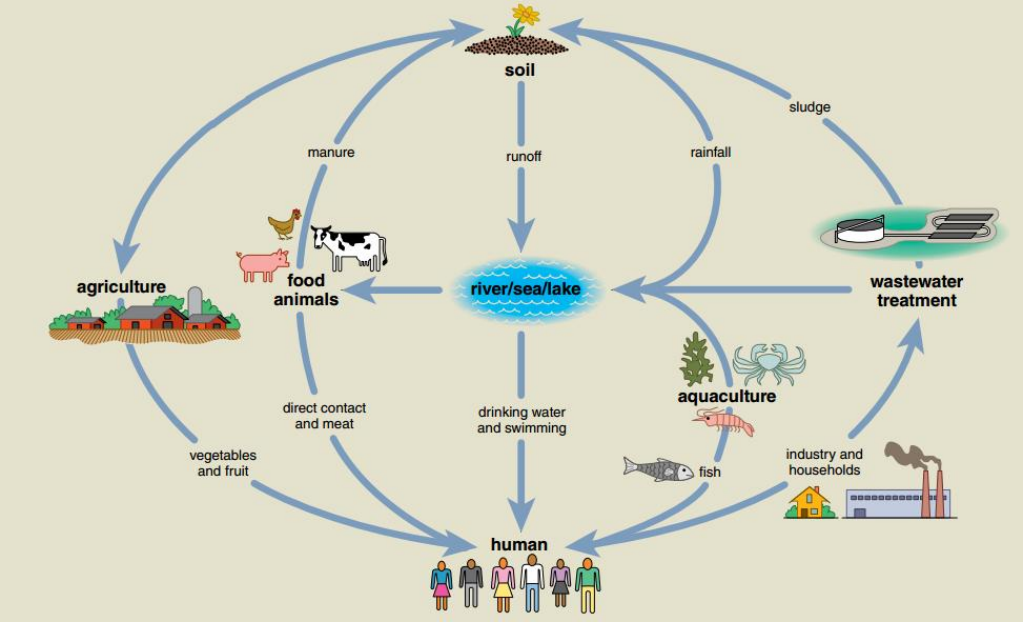

Figure 1.4. Epidemiology of antibiotic resistance: representation of direct and indirect interactions between soil, water, humans, food animals, agriculture, wastewater treatment plants, aquaculture and industry/households (Dantas and Sommer, 2014).

Recently, the class 1 integrase gene (*intI1*) was suggested as a good biopollution marker, due to its multiple attributes: (1) it is frequently associated to genes conferring resistance to antibiotics, disinfectants and heavy metals; (2) it can be detected in a variety of bacteria; (3) its abundance can fluctuate rapidly because its host cells present rapid generation times; (4) it can travel between bacteria by horizontal gene transfer; (5) a single DNA sequence variant of *intI1* can now be found on a wide diversity of xenogenetic elements, which comprise of complex mosaic DNA elements fixed through the action of

human selection. This gene can thus be used to track anthropogenic influences in several bacteria and environments (Gillings et al., 2015).

Although pinpointing specific pitfalls of resistance is complex, it is understandable that the misuse of antibiotics in human and veterinary medicine, particularly food production, is fueling the mobilization, evolution and spread of antibiotic resistance genes (Veldman et al., 2011; Dantas and Sommer, 2014). Over the years, the study of antibiotic resistance has been focused on the investigation of bacterial pathogens and on their direct consequences to human health (Davies and Davies, 2010). Considering that the origin of antibiotic resistance is within the environmental microbiota, it is extremely important to study resistance in non-clinical habitats in order to fully understand the cycle of acquisition of resistance by human pathogens. Thus, as we will observe during this thesis, the investigation of antibiotic resistance should not be confined to clinical-associated ecosystems.

1.3. Antibiotic resistance in Gram negative bacteria

"In Gram negatives more is believed than known"

Robert Bonomo, 2012

1.3.1. Antibiotic resistance mechanisms

Just as there are a number of different ways for bacteria to acquire an antibiotic resistance gene, the genes and respective proteins can themselves present a number of different strategies to encode resistance.

Globally, the genes that grant antibiotic resistance can be vaguely distributed into four main groups, each with their own distinctive mechanism for evading antibiotic exposure: 1) inhibition of antibiotic uptake due to permeability changes in the bacterial cell wall; 2) active efflux of the antibiotic from the microbial cell throughout activation of efflux pumps; 3) enzymatic modification of the antibiotic by inactivating or modifying enzymes; and 4)

modification or evasion of the drug target to prevent the recognition and association with the antibiotic (Figure 1.5) (Wright, 2010; Schmieder and Edwards, 2012; Blair et al., 2015).

In normal conditions, the flow of molecules into the cell is ensured by complex membrane proteins, known as OMPs (Outer Membrane Proteins), which provide channels for the entrance of a variety of molecules into the cell. The entrance of this molecules, which include hydrophilic antibiotics, such as aminoglycosides, β-lactams, glycopeptides, and colistin is based on their charge, shape, and size (Delcour, 2009). It is now known that the existence of a lower number of functional porins within the membrane can be due to amino acid substitutions that result in the alteration of the protein's framework, or to a decrease in the protein's regulation (Delcour, 2009). Thus, antibiotic resistance may occur through a decrease in the permeability of the outer membrane of Gram negative bacteria, resulting in the inhibition of antibiotic uptake (Delcour, 2009) (Figure 1.5). For instance, loss of function in the OprD (D2) porin causes imipenem resistance in *P. aeruginosa* (Yan et al., 2014). In *K. pneumoniae*, Ompk35, Ompk36 and OmpK37 have been frequently associated with antibiotic resistance, particularly to β-lactam antibiotics (Papagiannitsis et al., 2013).

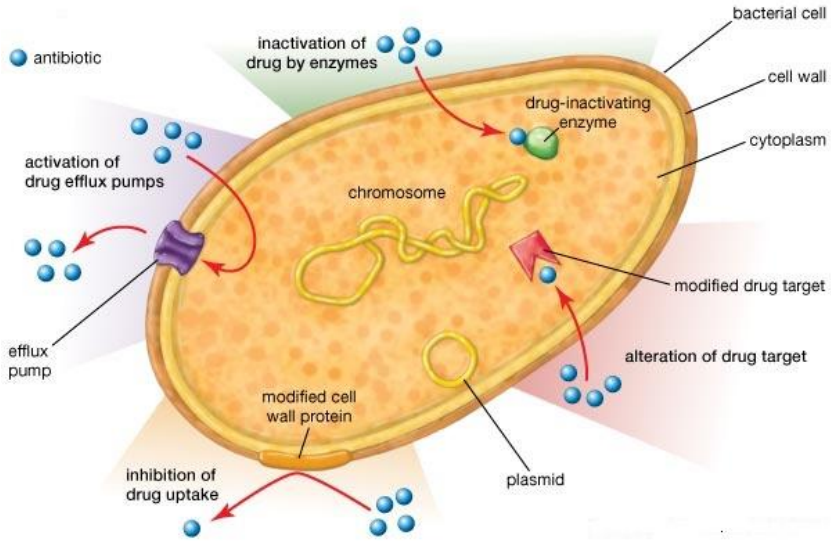

Figure 1.5. Mechanisms of antibiotic resistance (Hassler, 2015).

Efflux pumps are transport proteins involved in the extrusion of toxic substrates, including many classes of clinically relevant antibiotics from within the cell into the external environment (Figure 1.5) (Li et al., 2015b). Resistance is frequently caused by an increase in the synthesis of these proteins, due to the appearance of mutations in transcriptional

regulators, leading to an increased efflux of antibiotics (Blair et al., 2014; Blair et al., 2015). An example of this mechanism has been observed for tetracycline, an agent that is not only used to treat a wide variety of infections, but is also used in the farming of crops and livestock (Chopra and Roberts, 2001). Resistance to this class of antibiotics can emerge through the presence of tetracycline efflux-encoding genes, such as *tetA*, which code for proteins that are responsible for the active transport of the antibiotic. However, many efflux pumps are able to transport a wide range of structurally dissimilar substrates and are known as multidrug resistance efflux pumps. There are well-studied examples of these multidrug resistance efflux mechanisms, such as the AcrAB-TolC efflux pump, which mediate resistance to antibiotics and other toxic compounds in many bacteria (Ruiz and Levy, 2014; Li et al., 2015b).

The third group of antibiotic resistance mechanisms refers to the modification or inactivation of the antibiotic molecules by enzymes, rendering it inactive (Figure 1.5). The best known example of this strategy is used against β-lactam antibiotics, the most widely prescribed and diverse chemical class of antibiotics (Babic et al., 2006). β-lactam antibiotics inhibit the enzymes that synthesize and remodel the bacterial cell wall, which are essential for the cell during division and growth. Resistance towards those antibiotics is frequently conferred by β-lactamases, enzymes that cleave the β-lactam ring, making the antibiotic ineffective in inhibiting the cell wall proteins (Blair et al., 2015). This process is actually the most relevant antibiotic resistance mechanism in Gram negative bacteria (Bush, 2010). Enzymatic modification also constitutes a common type of resistance mechanism in other antibiotic classes, such as aminoglycosides and chloramphenicol (van Hoek et al., 2011; Blair et al., 2015).

Lastly, bacteria are also able to alter the drug target through the acquisition of mutations in the genes that encode the target of the antibiotic, compromising the ability of the antibiotic to effectively bind it (Figure 1.5). Resistance can thus emerge by changes to that structure, preventing efficient antibiotic binding, but still enabling it to carry out its normal function (van Hoek et al., 2011; Blair et al., 2015). For instance, point mutations in the *gyr* and *par* genes, which encode DNA topoisomerases, modify the binding efficiency of quinolone antibiotics, reducing their action. In fact, multiple point mutations may occur, particularly within the quinolone resistance determining region (QRDR) of DNA topoisomerase II (*gyrA*, *gyrB*) and IV (*parC* and *parE*) encoding genes, which frequently lead to high levels of resistance (Fàbrega et al., 2009).

1.3.2. Intrinsic resistance

Bacteria can be intrinsically resistant to certain antibiotics but can also acquire resistance by mutation and by horizontal gene transfer (van Hoek et al., 2011). The intrinsic resistance of a bacterial species is the capability to evade the action of a specific antibiotic, as a result of innate genetic, structural, and/or functional characteristics (Cox and Wright, 2013; Blair et al., 2015). The simplest example of intrinsic resistance derives from the absence of a target for a specific antibiotic, thought it can also emerge from more complex mechanisms (Figure 1.6).

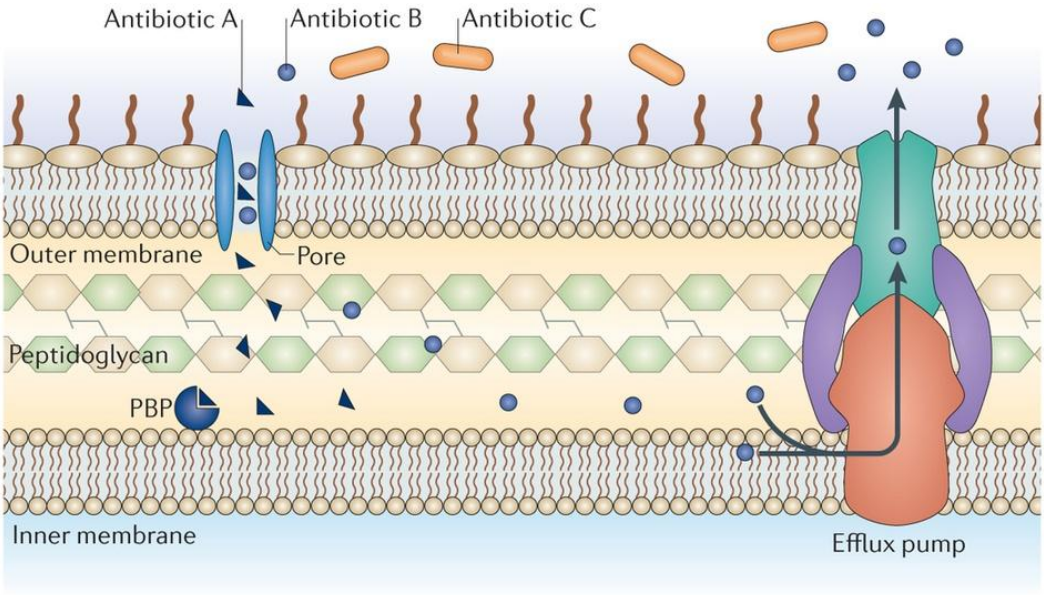

Figure 1.6. Overview of intrinsic resistance mechanisms against β-lactam antibiotics targeting a penicillin-binding protein (PBP). Antibiotic A can enter the cell via an outer membrane protein, reach its target and inhibit peptidoglycan synthesis; antibiotic B can also enter the cell via one of these proteins, but it is efficiently removed by the action of an efflux pump; antibiotic C cannot cross the outer membrane and so is unable to access the target (Blair et al., 2015).

For instance, the lipopeptide daptomycin is active against Gram positive but it is not against Gram negative bacteria. This is due to an intrinsic difference in the composition of the cytoplasmic membrane: Gram negative bacteria have a lower proportion of anionic phospholipids in the cytoplasmic membrane than Gram positives, which reduces the efficiency of the Ca^{2+}-mediated insertion of daptomycin into the cytoplasmic membrane that is required for its antibacterial activity (Blair et al., 2015). In fact, the intrinsic

resistance of Gram negative bacteria to many compounds is due to an inability of these agents to cross their outer membrane. Table 1.2 represents an overview of some of the main known bacterial intrinsic resistance mechanisms.

Table 1.2. Examples of known intrinsic resistance mechanisms in bacteria.

Bacteria	Antibiotic	Mechanism
All Gram negative bacteria	Vancomycin and daptomycin	Vancomycin and daptomycin are unable to penetrate the Gram negative outer membrane
All Gram positive bacteria	Aztreonam	Lack of penicillin binding proteins which can effectively bind aztreonam
Klebsiella spp.	Penicillins	Chromossome-encoded β-lactamase inactivates the antibiotic before it reaches the target
Stenotrophomonas maltophilia	Imipenem	Chromossome-encoded metallo-β-lactamase inactivates the antibiotic before it reaches the target
Pseudomonas aeruginosa	Sulphonamides, tetracycline, chloramphenicol	Ineffective intracellular concentrations of antibiotic due to lack of uptake
Enterococcus spp.	Aminoglycosides	Limited uptake of aminoglycosides by proteins of electron transport chain

Intrinsically resistant bacteria have emerged as a relevant health problem in the last years. Those bacterial species, some of them with environmental origins but that now act as opportunistic pathogenic agents, present naturally low level susceptibility to several clinically relevant antibiotics (Cox and Wright, 2013). For a long time, and from a clinical point of view, it has been proposed that intrinsic resistance is simply the outcome of the impermeability of bacterial cell membranes, the action of multidrug efflux pumps or even the lack of appropriate targets for a given antibiotic. However, it is known that all bacterial genomes encode multidrug resistance efflux pumps, and that many species carry chromosome encoded antibiotic-modifying proteins, even though they are not classified as intrinsic resistant (Olivares et al., 2013). Recent studies suggest that the species specific antibiotic susceptibility depends on the concerted activity of numerous other elements besides classical chromosomal resistance genes, belonging to all functional categories, which has been named as intrinsic resistome (Olivares et al., 2013). Thus, understanding

the genetic basis of intrinsic resistance, and hence the spectrum of activity of an antibiotic towards specific bacterial species, can guide the development of new combinations of agents with improved activity.

1.3.3. Acquired resistance

Acquired resistance consists in the result of a series of genetic changes that include the horizontal acquisition of mobile antibiotic resistance genes[2] through the intervention of transferable genetic elements and the appearance of mutations in structural or regulatory housekeeping genes (van Hoek et al., 2011). In the latter example, mutations are often associated with the target of a specific antibiotic class, such as *gyrA* in fluoroquinolones (Fàbrega et al., 2009), *ramR* in tetracyclines (Wang et al., 2015b), or *ftsZ* in β-lactam antibiotics (Barbosa et al., 2011). It should however be noticed that many mechanisms may not directly confer high levels of resistance to the antibiotic and additional mechanisms may be required (Blair et al., 2015).

In this section we will briefly discuss the mode of action of some relevant antibiotic classes, as well as the major acquired resistance mechanisms in Gram negative bacteria that are currently undermining human healthcare facilities, veterinary clinics, or industrial farming units.

1.3.3.1. Acquired resistance to β-lactams

The β-lactam antibiotics, which encompass penicillins, cephalosporins, cephamycins, carbapenems, monobactams, and β-lactamase inhibitors, constitute the most successful class of antibiotics ever developed (Bush and Jacoby, 2010; Blair et al., 2015). Their high efficacy and low toxicity makes them the most frequently prescribed antibiotics for humans and animals, generating a prevailing selection pressure for genes encoding resistance in environments proximal to human activity. β-lactam antibiotics are composed

[2] The term mobile antibiotic resistance gene or mechanism will be used to define any antibiotic resistance gene or mechanism that is frequently, or that have been previously acquired by horizontal gene transfer, and/or disseminated by mobile genetic elements.

either by an isolated β-lactam ring in monobactams or by bicyclic ring structures in the other classes. The antibacterial properties of the diverse β-lactams differ according to the variability of the side chains (van Hoek et al., 2011). They work through the inhibition of the cell wall synthesis by binding to penicillin-binding proteins (PBPs), and by interfering with the structural cross linking of peptidoglycan, preventing terminal transpeptidation in the bacterial cell wall (van Hoek et al., 2011). In consequence, the bacterial cell wall is weakened, resulting in the lyses of the cell. However, despite the success of β-lactam antibiotics in the treatment of infectious diseases, the respective mechanisms of resistance are unprecedented (Bush and Jacoby, 2010).

Resistance to β-lactam antibiotics can be acquired by the alteration of the target (PBPs), particularly in Gram positive bacteria. Mechanisms for the active efflux of the antibiotic and decrease of its uptake by porins also contribute to resistance, though often in combination with complementary mechanisms, such as the production of β-lactamases. Indeed, it is the enzymatic inactivation of the antibiotic by β-lactamases that constitutes, by far, the most significant antibiotic resistance mechanism against β-lactam antibiotics, particularly in Gram negative bacteria, for which reason it will be further explored in this section (Drawz and Bonomo, 2010; van Hoek et al., 2011).

The clinical importance and complexity of the β-lactamases created the need to cluster them in coherent groups, according to their molecular homology and hydrolysis profile (Table 1.3).

Molecular classes A, B, C, and D, define an enzyme according to the amino acid sequence and conserved motifs: Ambler classes A, C, and D include the β-lactamases with serine at their active site, while Ambler class B β-lactamases are metallo-enzymes who require zinc as a metal cofactor for their catalytic activities (Ambler, 1980). In contrast, functional groups 1, 2, and 3 of Bush and Jacoby (Bush and Jacoby, 2010) are used to assign a clinically useful description to a group of enzymes, with subgroups designated according to substrate and inhibitor profiles. Groups 1 and 2 include all serine-β-lactamases, while members of group 3 consist of metallo-β-lactamases (MBL) (Table 1.3) (Bush, 2010).

Table 1.3. Classification schemes for the major families of β-lactamases of clinical importance in Gram negative bacteria (adapted from Bush and Jacoby, 2010).

Ambler Class	Functional group or subgroup[1]	Enzyme families	Preferred substrates and inhibitor profile	Representative enzymes
A	2b	TEM SHV	Penicillins, early cephalosporins (inhibited by β-lactamase inhibitors)	TEM-1, TEM-2, TEM-13 SHV-1, SHV-11, SHV-89
	2be (ESBL)	TEM SHV	Extended-spectrum cephalosporins, monobactams (inhibited by β-lactamase inhibitors)	TEM-10, TEM-24 SHV-12
		CTX-M		CTX-M-1 to CTX-M-170
		VEB		VEB-1 to VEB-16
		PER		PER-1 to PER-8
	2br (IRT/IRS)	TEM SHV	Penicillins (resistance to β-lactamase inhibitors)	TEM-30, TEM-31 SHV-72, SHV-84, SHV-107
	2ber (CMT)	TEM	Extended-spectrum cephalosporins, monobactams (resistance to β-lactamase inhibitors)	TEM-50, TEM-158
	2f	GES	Increased hydrolysis of carbapenems, oxyimino-β-lactams and cephamycins (variable resistance to β-lactamase inhibitors)	GES-2 to GES-27
		KPC		KPC-2 to KPC-24
		SME		SME-1 to SME-5
B	3a (MBL)	IMP	Broad-spectrum hydrolysis including carbapenems but not monobactams (resistance to β-lactamase inhibitors)	IMP-1 to IMP-53
		VIM		VIM-1 to VIM-46
		NDM		NDM-1 to NDM-16
C	1 (PMAβ)	CMY	Cephalosporins, cephamycins (resistance to β-lactamase inhibitors)	CMY-1 to CMY-135
		DHA		DHA-1 to DHA-23
	1e (ESAC)	CMY	Cephalosporins, cephamycins, with increased hydrolysis of ceftazidime. (resistance to β-lactamase inhibitors)	CMY-10, CMY-19, CMY-37
D	2d	OXA	Cloxacillin (variable resistance to β-lactamase inhibitors)	OXA-1, OXA-2, OXA-10
	2de (ESBL)	OXA	Cloxacillin, Extended-spectrum cephalosporins (variable resistance to β-lactamase inhibitors)	OXA-11, OXA-14, OXA-15
	2df (CHDL)	OXA	Cloxacillin, Carbapenems (variable resistance to β-lactamase inhibitors)	OXA-23, OXA-24, OXA-48, OXA-51, OXA-58

[1] Subgroups 2a, 2c, 2ce, 2e and 3b are missing in this table, because they represent enzymes not commonly encountered in clinical practice.

In addition, other designations are commonly used to define the functionality of specific subgroups based on the hydrolysis and inhibition profiles, such as extended-spectrum β-lactamases (ESBL), plasmid-mediated AmpC β-lactamases (PMAβ), metallo-β-lactamases, carbapenemem hydrolyzing class D β-lactamases (CHDL), and class A carbapenemases, among others (Table 1.3) (Jacoby, 2009; Grundmann et al., 2010; Poirel et al., 2010; Cantón et al., 2012).

Over 1500 chromosomal-encoded and plasmid-mediated β-lactamases are currently known (Bush and Jacoby, 2010). During the past 15 years, the accumulation of amino acid substitutions in parental TEM and SHV enzymes, as well as the first appearance of the CTX-M β-lactamases, lead to a concerning emergence of mobile antibiotic resistance genes encoding ESBLs (Cantón et al., 2012).

Moreover, PMAβs such as CMY or DHA, which derived from chromosome-encoded AmpC enzymes that are responsible for intrinsic resistance to β-lactam antibiotics in many Gram negative species, have also caught people's awareness in the last 10 years. Overall, the above mentioned β-lactamases have settled, and are now prevalent not only in nosocomial and community settings (Cantón et al., 2012; Mata et al., 2012; Nicolas-Chanoine et al., 2014), but also in animals of many origins (Winokur et al., 2001; Ahmed et al., 2007; Ewers et al., 2012; Belas et al., 2014).

Nowadays, the worldwide emergence of resistance to carbapenems in Gram negative bacteria, particularly *Enterobacteriaceae*, constitutes an important growing public health threat (Hawkey, 2015). Sporadic outbreaks or endemic situations with carbapenem nonsusceptible isolates are now often described in hospitals of many countries, and are also starting to cause community acquired infections. Acquired class A (KPC), class B (IMP, VIM, NDM), or class D (OXA-48) carbapenemases, are the most important determinants sustaining resistance to carbapenems, in addition to all the remaining β-lactams (Nordmann and Poirel, 2014). These carbapenem hydrolyzing β-lactamases are rapidly emerging in Europe, where countries such as Italy and Greece have already described endemic situations (Figure 1.7) (Albiger et al., 2015; Hawkey, 2015).

Since February 2013 (Glasner et al., 2013), when Portugal only reported sporadic cases of carbapenemases occurrence, descriptions of OXA-48, VIM, IMP, NDM and predominance of KPC have been reported in the country (Manageiro et al., 2014; Manageiro et al., 2015a; Pires et al., 2016). Overall, β-lactamase-encoding genes are frequently associated with mobile genetic structures that are often shared by other antibiotic resistance determinants, further enhancing their success (El Salabi et al., 2013).

The detection of β-lactamases in remote Alaskan soils and in 30,000-year-old Beringian permafrost sediments, as well as their constitutive chromosomal production by many environmental bacteria, suggests that they are ubiquitous in the environment, and are thus likely to spread upon appropriate selective pressure (D'Costa et al., 2011; Olivares et al., 2013).

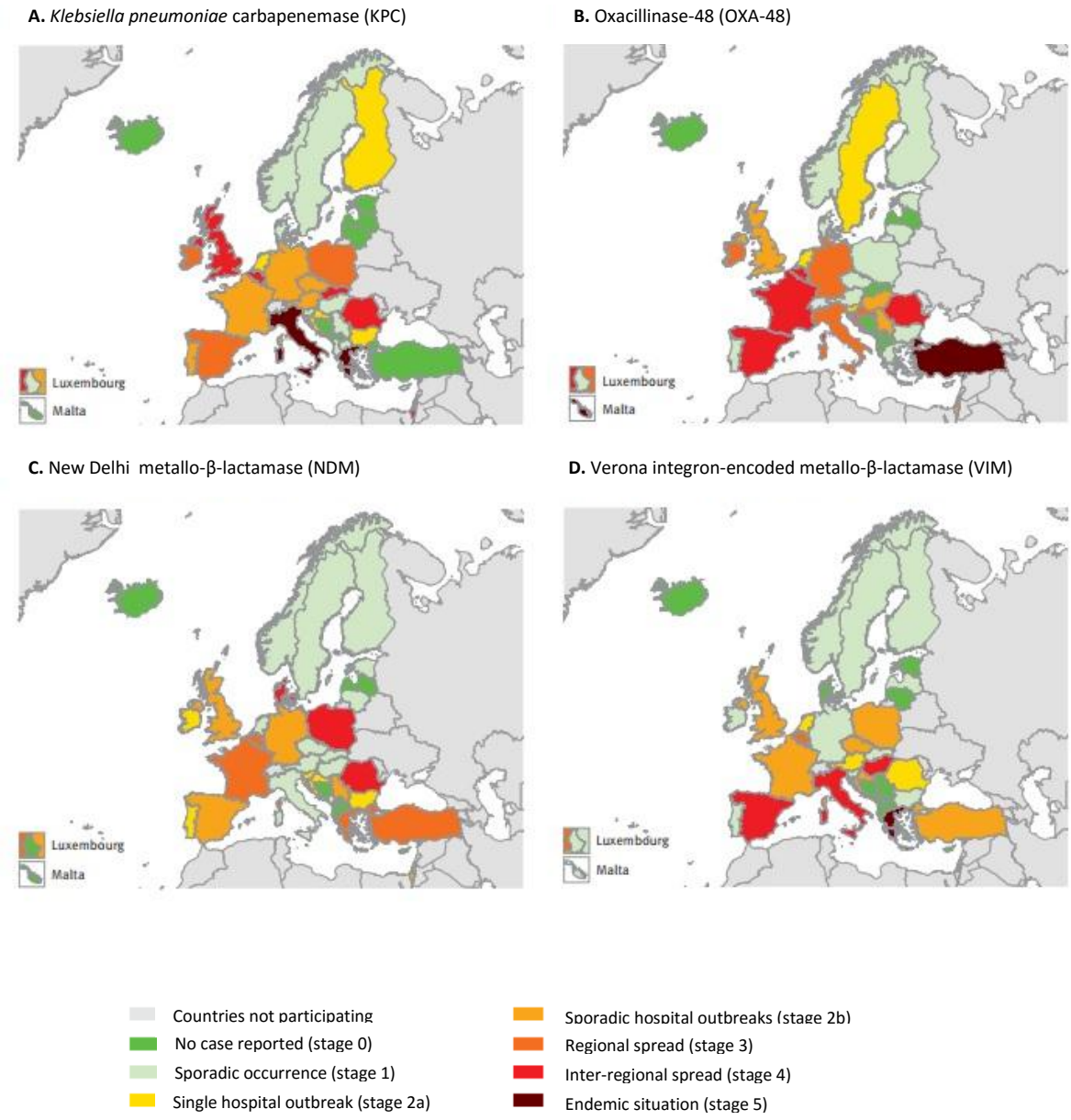

Figure 1.7. Occurrence of carbapenemase-producing *Enterobacteriaceae* by type of carbapenemases in 38 European countries based on self-assessment by the national experts, May 2015 (adapted from Albiger et al., 2015).

Considering that few reports exist on mobile carbapenemases-encoding genes from animal and environmental origin (Woodford et al., 2014; Morrison and Rubin, 2015), the surveillance of such isolates is essential to avoid their spread.

1.3.3.2. Acquired resistance to quinolones

Quinolones are broad-spectrum antibiotics that have been used in medical practice for the treatment of severe or resistant infections (van Hoek et al., 2011). This class of antibiotics is fully synthetic and used widely in both human and veterinary medicine. The second generation of quinolones arose when it became clear that the addition of a fluoride atom at position 6 of a quinolone molecule, creating a fluoroquinolone, greatly enhanced its biological activity (Fàbrega et al., 2009; Rodríguez-Martínez et al., 2011).

Quinolones and fluoroquinolones inhibit the action of DNA topoisomerases II (commonly designated by DNA gyrase) and IV, two enzymes essential for bacterial DNA replication and cell division. DNA topoisomerases consist of tetrameric enzymes composed of two subunits each: GyrA-GyrB and ParC-ParE, respectively. The four genes coding for the subunits of these enzymes (*gyrA*, *gyrB*, *parC* and *parE*) constitute the targets for mutations associated to bacterial resistance to both quinolones and fluoroquinolones (van Hoek et al., 2011). The region where mutations arise is a short DNA sequence known as the quinolone resistance-determining region (QRDR) (Rodríguez-Martínez et al., 2011). Mutations in the QRDR of these genes, resulting in amino acid substitutions, alter the target protein structure and subsequently the fluoroquinolone-binding affinity of the enzyme, leading to antibiotic resistance. Besides the appearance of those mutations, the other chromosome-encoded acquired resistance mechanisms comprise the up-regulation of naturally occurring efflux pumps and, to a less extent, porin loss (Robicsek et al., 2006a; Fàbrega et al., 2009).

The plasmid-mediated quinolone resistance (PMQR) includes three main classes of resistance determinants. The *qnr* genes encode a protein that protects the DNA gyrase and type IV topoisomerase from quinolone inhibition. Currently, six families of *qnr* genes have been reported, some with more than one variant (Table 1.4): *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS* and *qnrVC1* (Poirel et al., 2012). The second type of plasmid located quinolone resistant gene is a variant of the aminoglycoside resistance gene *aac(6')-Ib*, which is designated *aac(6')-Ib-cr.* This gene encodes the aminoglycoside acetyltransferase AAC(6')-Ib-cr, which

harbors two essential amino acid changes in positions 102 and 179 that guarantee the ability to acetylate certain fluoroquinolones (Robicsek et al., 2006b) (Table 1.4). The third class of mobile fluoroquinolone-resistance genes includes the *oqxAB* and *qepA* efflux systems, which encode transporters that can actively export fluoroquinolone molecules outside the cell (Table 1.4). Carriage of PMQR-encoding genes often confers modest increases in the Minimum Inhibitory Concentration (MIC) of fluoroquinolones (Strahilevitz et al., 2009). Recently, resistance to quinolones and fluoroquinolones has been increasingly reported among human but also veterinary isolates, very likely as a consequence of the great usage of those antibiotics (Tamang et al., 2011). Current studies have identified the environment as a reservoir of PMQR genes, with farm animals and aquatic habitats being significantly involved (Adachi et al., 2013; Kaplan et al., 2013; Börjesson et al., 2015; Varela et al., 2015). In addition, the putative origin of the *qnrA* genes has been attributed to the waterborne species *Shewanella* sp., which suggests that the aquatic environment might constitute the original source of, at least, some PMQR genes (Poirel et al., 2005).

Table 1.4. First descriptions of plasmid-mediated fluoroquinolone resistance (PMQR) encoding genes from different families.

Group	Gene	Species	Reference
Efflux pumps	*qepA*	*Escherichia coli*	Yamane et al., 2007
	oqxAB	*Escherichia coli*	Hansen et al., 2004
Pentapeptide repeat protein family	*qnrA1*	*Klebsiella pneumoniae*	Tran and Jacoby, 2002
	qnrB1	*Klebsiella pneumoniae*	Jacoby et al., 2006
	qnrC	*Proteus mirabilis*	Wang et al., 2009
	qnrD	*Salmonella enterica*	Cavaco et al., 2009
	qnrS1	*Shigella flexneri*	Hata et al., 2005
	qnrVC1	*Vibrio cholerae*	Fonseca et al., 2008
Acetyltransferase	*aac(6')-Ib-cr*	*Escherichia coli*	Robicsek et al., 2006b

1.3.3.3. Acquired resistance to other relevant antibiotic classes

Chloramphenicol consists of a highly specific and effective inhibitor of protein synthesis through its affinity for the peptidyltransferase of the 50S ribosomal subunit, preventing peptide chain elongation and inhibiting protein biosynthesis (Blair et al., 2015). The first

and still most frequently detected mechanism of bacterial resistance to chloramphenicol is the enzymatic inactivation of the drug via different types of chloramphenicol acetyltransferases (*cat*). Besides the existence of chloramphenicol-inactivating enzymes, there are also reports of other chloramphenicol resistance mechanisms, such as inactivation by phosphotransferases, mutations of the target site, permeability barriers, and active efflux systems. Of the latter mechanisms, the plasmid-mediated efflux pumps *cmlA* and *floR* are the most commonly described. Chloramphenicol analogs including the fluorinated derivative florfenicol have a similar spectrum of activity but differ in their therapeutically targets: while chloramphenicol is approved for human use, florfenicol is mainly recommended for the treatment of bovine respiratory pathogens (van Hoek et al., 2011).

Tetracyclines were the first major group to which the term "broad spectrum" was applied. They exhibit antibiotic activity against a wide range of microorganisms and sterically block aminoacyl-tRNA binding with the bacterial ribosome, inhibiting protein synthesis (van Hoek et al., 2011). Resistance to tetracyclines occurs through five types of mechanisms that include the production of ribosomal protection proteins (RPPs), active efflux of tetracycline from the cell, enzymatic inactivation of the antibiotic, decrease of membrane permeability, and amino acid alteration of the antibiotic targets. However, tetracycline resistance is most often due to the acquisition of genes coding for energy dependent efflux pumps or for proteins that protect the ribosomes from the action of this antibiotic. There are currently more than 40 different acquired tetracycline resistance determinants documented: *tet* (tetracycline resistance) and *otr* (oxytetracycline resistance) genes. There are reports indicating increasing numbers of Gram negative bacteria carrying typical Gram positive *tet* genes, changing our perspective in what may be relevant to screen when dealing with tetracycline resistance (Chopra and Roberts, 2001; Roberts, 2003).

Aminoglycosides act primarily by impairing bacterial protein synthesis through binding to the 30S subunit of prokaryotic ribosomes. This does not prevent formation of the initiation complex of peptide synthesis, but perturbs the elongation of the nascent chain by impairing the proofreading process that controls translational accuracy (Davies, 2006). Several aminoglycoside resistance mechanisms have been recognized to date: active efflux, decreased permeability, ribosome alteration, and inactivation of the antibiotic by aminoglycoside-modifying enzymes (Blair et al., 2015). The major encountered aminoglycoside resistance mechanism is enzymatic modification of the antibiotic. The proteins responsible for this process are classified into three major classes according to

the type of modification: AAC (acetyltransferases), ANT (nucleotidyltransferases or adenyltransferases), and APH (phosphotransferases) (Ramírez and Tolmasky, 2010). The number of aminoglycoside modifying enzymes identified to date, as well as the genetic environments where the coding genes are located is impressive, and there is virtually no bacteria that is unable to support enzymatic resistance to aminoglycosides (van Hoek et al., 2011).

Sulphonamides belong to the oldest introduced antibiotic drug, first used in 1932. A number of different sulphonamides have been developed of which the most commonly used are sulfamethoxazole and the combination of trimethoprim with sulfamethoxazole (co-trimoxazole). Both sulphonamides and trimethoprim are involved in the pathway leading to biosynthesis of folic acid that is required for thymine production and bacterial cell growth (Huovinen, 2001). Those drugs competitively inhibit the enzymes dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR), encoded by genes *folP* and *folA*, respectively. Low level chromosomal sulfonamide resistance can occur through the existence of drug-resistant variants of the chromosomal *fol* genes encoding the bacterial DHFR and DHPS. Currently, two different sets of plasmid-borne antibiotic resistant variants of the DHPS/DHFR-encoding genes guarantee the resistance to sulphonamides and trimethoprim (Roberts, 2002). Firstly, three plasmid-borne drug-resistant variants of DHPS enzymes are known: *sul1*, *sul2* and *sul3*. Secondly, the plasmid-mediated DHFRs consist of six families of mobile *dfr*, which include *dfrA* and *dfrB* genes in Gram negative bacteria and *dfrC*, *dfrD*, *dfrG*, and *dfrK* in Gram positive bacteria (Roberts, 2002).

Polymyxin E (colistin) is a last resource group of antibiotics that can be used for the treatment of infections caused by multidrug resistant Gram negative bacteria, and, nowadays, is often the only therapeutic option available (Neuner et al., 2011). Their action on Gram negative bacteria shows a detergent-like effect. Initial binding carried out between the polycationic ring of colistin to cell envelope components, causes the displacing of the calcium and magnesium ions from the phosphate groups of membrane lipopolysaccharides, leading to disruption of the outer membrane (Bialvaei and Samadi Kafil, 2015). Until November 2015, resistance was based in chromosomal mutations but has never been reported via horizontal gene transfer. However, during a routine surveillance project on antimicrobial resistance in commensal *E. coli* from food animals in China, the new plasmid-mediated colistin resistance gene *mcr-1* has been identified in pigs (Liu et al., 2016). Only three months after the first report, and before the clarification of the mechanism of action itself, the *mcr-1* gene has already been detected in animals,

humans and food from animal origin in Asia, Africa and Europe, anticipating the dawn of the post-antibiotic era (Hasman et al., 2015; Webb et al., 2015; Stoesser et al., 2016; Yao et al., 2016).

All of the above mentioned antibiotics are used in human and veterinary medicine, particularly in the farming of food-producing animals. Moreover, chloramphenicol, aminoglycosides, tetracycline, sulfonamide, trimethoprim, and by now, colistin acquired resistance genes can be found in a variety of bacteria isolated from humans, animals, and the environment (Davies and Davies, 2010; Berendonk et al., 2015; Caniça et al., 2015). The repeated presence of antibiotic resistance genes such as *cat*, *tet*, *aadA*, *drfA* and *sul* in wide disseminated class 1 and class 2 integrons may partially explain their distribution among many bacterial species, and their persistence in settings that virtually do not harbor significant antibiotic selective pressure.

1.4. Omic approaches in the study of antibiotic resistance

"I have not failed. I've successfully discovered 10,000 things that won't work"

Thomas Edison, 1898

Microbiology has experienced a transformation during the last three decades that has altered the perspectives on how to study microorganisms. The applications of *omic* approaches were enhanced by the appearance of high-throughput technologies such as Next Generation Sequencing (NGS) and Mass spectrometry (MS), which constitute valuable tools for the massive and rapid analysis of genomes and proteomes, respectively (Zhang et al., 2010; Franzosa et al., 2015).

Antibiotic resistance aims to study the genetic and functional processes that allow bacteria to survive the exposure to antimicrobial agents. Such a multi-dimensional and detailed investigation requires large-scale experiments involving whole genetic, structural, or functional components. Thus, the recent *omic* technologies are suitable to pursue these types of investigations since they are primarily aimed at the universal detection of genes (genomics), mRNA (transcriptomics), and proteins (proteomics) in a single biological

sample, towards the understanding of the complete phenotype (phenomics) (Figure 1.8) (Franzosa et al., 2015). Other *omic* technologies are available but in this thesis we will focus on the relevance and applications of the analysis of genomes and proteomes in the scope of antibiotic resistance.

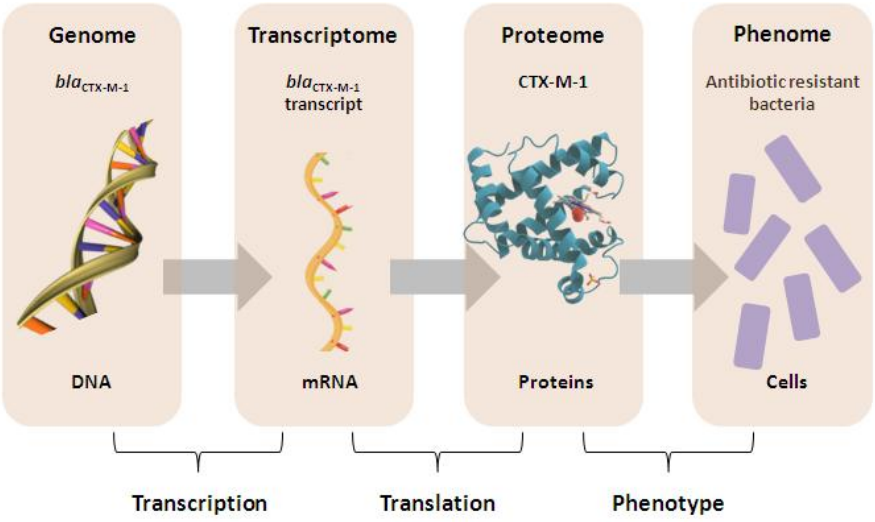

Figure 1.8. Cascade of *omic* approaches used to decode genotype–phenotype interactions in a $bla_{CTX-M-1}$-harboring isolate.

1.4.1. Genomics

Genomics is the study of all the genes that are present in a living organism, offering an integrated view of all possible gene products. For Gram negative bacteria, the investigation of genomics has seriously increased in 1995 with the publication of the first bacterial genome sequence obtained through Sanger sequencing (Fleischmann et al., 1995). Nearly a decade later, the dawn of the first high-throughput sequencing technologies [now commonly designated Next Generation Sequencing (NGS)] marked the beginning of an era of significant progress in the ease and cost of whole genome sequencing (WGS), with the delivery of bacterial genome sequences taking place in days rather than months or years (Loman et al., 2012a).

NGS platforms can be divided into two broad groups depending on the kind of template used for the sequencing reactions (Figure 1.9). The earliest and currently most widely used platforms, which include machines from Roche, Thermofisher Scientific (former Life

Technologies), Illumina and, more recently, Oxford Nanopore, depend on the production of libraries of clonally amplified templates. These are produced through amplification of immobilized libraries made from a single DNA molecule in the initial sample. More recently, we have seen the arrival of single-molecule sequencing platforms (Pacific BioSciences), which determine the sequence of single molecules without amplification (Figure 1.9) (Loman et al., 2012a; Loman et al., 2012b). Within these platforms, there is considerable variation in performance (throughput, read length and error rate), and in factors affecting usage, such as cost and run time.

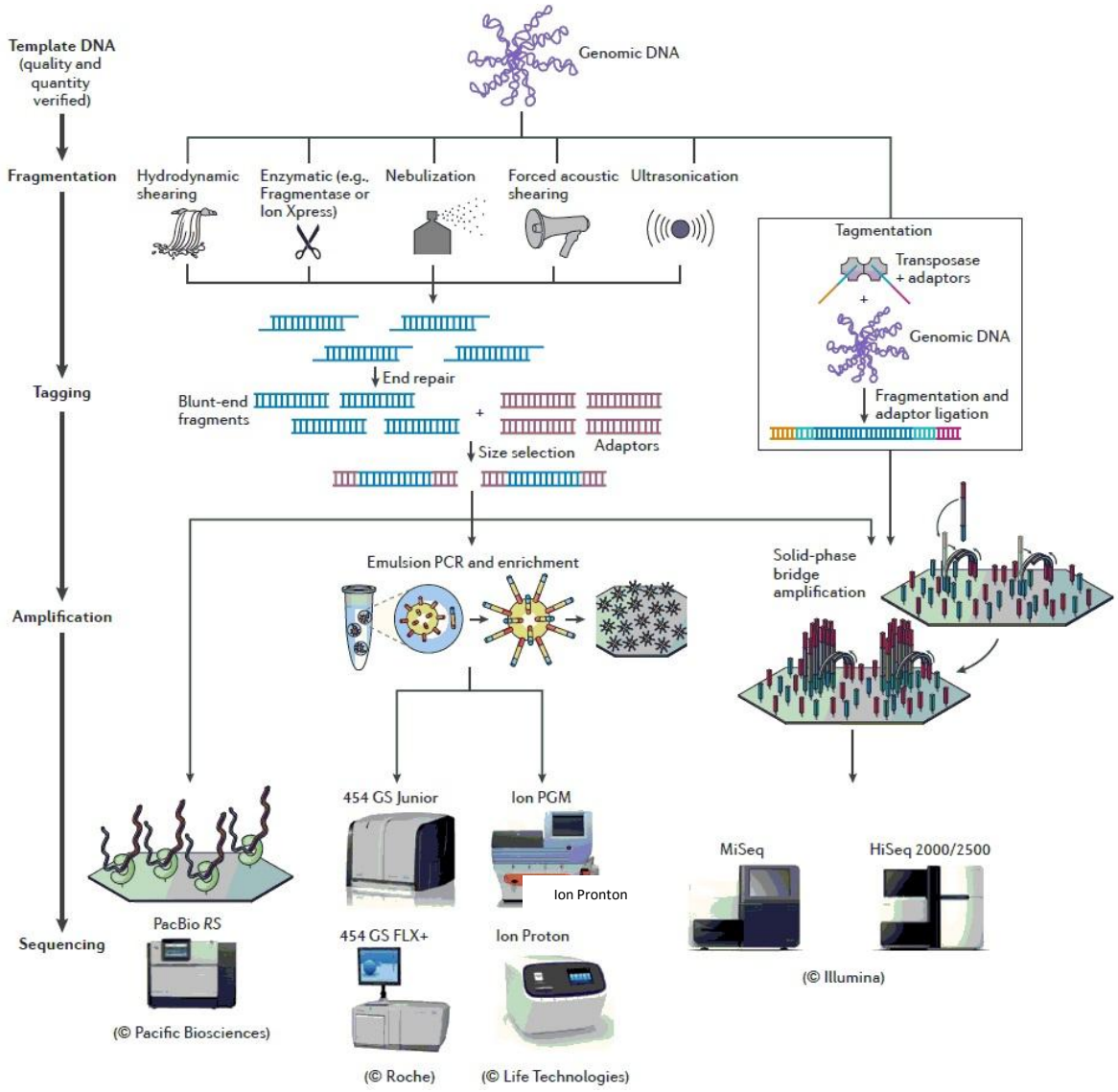

Figure 1.9. Main high-throughput sequencing platforms available to microbiologists and the associated sample preparation and template amplification procedures (adapted from Loman et al., 2012a).

Although the main bottleneck in genomics is still the bioinformatics analysis, the need for rapid and specialized investigation of bacterial genomes led to the emergence of several user-friendly software and web-tools that allow the easy retrieval of specific data (Larsen, 2013; McArthur and Wright, 2015). WGS has become an invaluable tool in the struggle against antibiotic resistance, and the comparative analysis of bacterial genomes is becoming crucial in the definition of Gram negative and positive bacteria associated disease in clinical contexts. Azarian et al. (2015) investigated a methicilin resistant *Staphylococcus aureus* outbreak in a neonatal intensive care unit using WGS, and suggested that the comparison of genome-wide single nucleotide polymorphisms may identify epidemiologically distinct isolates, which appear homogenous when evaluated using conventional typing methods.

Moreover, the growing availability of either draft or complete genomes of microorganisms recovered among the most varied sources will certainly enable broad comparative genomic analyses that may serve a greater epidemiological purpose (Azarian et al., 2015).

This ongoing revolution in genomic science and sequencing technology has strongly impacted environmental genomics, providing increasingly comprehensive data for microorganisms that cannot be readily cultivated under laboratory conditions. Metagenomic analysis offers such an alternative to traditional techniques because it does not require the cultivation of microorganisms, analyzing the genomic DNA from all the microbial community present in a sample. It allows the detection of antibiotic resistance genes in specific areas of interest that may have been altered by human activity, such as wastewater treatment plants and hospital settings (Garmendia et al., 2012). Metagenomics is currently being used for describing (descriptive metagenomics) the structure of microbial populations. Since most microorganisms cannot be cultured, the description of microbiota can be addressed by two complementary approaches: analysis of the composition of the microbiota of a certain habitat (e.g. soil, human gut) and tracking the presence and abundance of already known antibiotic resistance genes in different ecosystems (e.g. biopolutants). The first approach is used for broadly understanding the population present in a given ecosystem. The second is particularly useful for understanding the metagenome of the microbial community, evaluating the influence of environmental conditions in the detection of specific genes, such as antibiotic resistance genes (Garmendia et al., 2012; Franzosa et al., 2015). With few new antibiotics in the discovery pipeline, functional metagenomics can be useful for searching for new antibiotics without the need for culturing the producer microorganisms (Pehrsson et al., 2013). In this case, metagenomic microbial DNA is directly extracted from any

environment and cloned into a cultivable host strain. Metagenomic transformants harboring DNA fragments that encode antimicrobial peptides or antibiotic resistance mechanisms are then selected by subjecting the library of clones to susceptible strains or specific antibiotics, respectively (Schmieder and Edwards, 2012). Despite the relevance of the problems that need addressing, the number of studies on metagenomics and antibiotic resistance is still low. More studies are required in this field to better understand antibiotic resistance in specific microbial communities and to find novel compounds with antimicrobial activity (Garmendia et al., 2012).

1.4.2. Proteomics

The study of proteomes complements other available *omic* approaches by providing data on the nature of the final gene product - the protein. Specifically, the analyses of a single proteome can provide information not available by other methods on the occurrence of post-translational modifications, protein subcellular localization, and protein regulation (Burchmore, 2014). Nowadays, MS-based quantitative proteomics constitute the prevailing technology that is capable of addressing such questions. The recent progresses noticed in overall instrument sensitivity, speed of data acquisition, and the success obtained with the interface between mass spectrometry and liquid chromatography (LC–MS/MS) made gel-free approaches very popular, allowing the direct quantitative comparison of different proteomes (Vranakis et al., 2014).

Although this protein-faced *omic* approach has been extensively used to investigate human diseases, it has also been quite helpful in the analyses of microbial gene expression. The parallel evolution of proteomics and genomics allowed the study of proteins to be supported by the availability of whole genome sequences of many bacteria, including pathogenic agents, boosting the identification of clinically important microbial proteins (Franzosa et al., 2015).

The first impact of proteomics in microbiology was its ability to generate proteome maps containing a detailed vision of the overall microbial gene expression. The major advantage of this approach was the possibility to compare catalogs of proteins expressed by an organism under different conditions, evaluating the differences in the end results. Thus, the proteomes of pathogenic bacteria growing *in vitro* provided an opportunity to carry out comparative studies under highly controlled conditions that have been specifically

used to identify correlations of specific proteins with pathogenicity, virulence and antibiotic resistance (Vranakis et al., 2014).

For instance, Lee et al (2012) used a proteomic approach to demonstrate that *K. pneumoniae* secretes outer membrane vesicles (OMVs), which in turn induce the innate immune response in hosts. This study revealed that 159 different proteins were associated with *K. pneumoniae* OMVs (Lee et al., 2012).

Bacteria come across several physiological and environmental challenges as they advance through their lifecycles. The survival of an organism depends, at least in part, on its ability to sense and respond to changes in its environment. To survive these extreme and rapidly changing conditions, bacteria must sense them and respond with appropriate alterations in gene expression and protein activity (Burchmore, 2014). Antibiotic exposure constitutes one of the most intense environmental stressors to which a response must be rapidly given. In fact, antibiotics have the ability to disturb some molecular aspects of the bacterial metabolism with deleterious consequences, which may become fatal depending on the time and concentration of exposure. If the individual microorganisms are not eliminated by exposure to the antibiotic, this challenge may be faced by adaptive responses that render the organism resistant. The mentioned changes may enable a pathogen to live long enough to survive antibiotic exposure or to adopt a niche where drug concentrations are lower. In the end, the ability to tolerate antibiotic exposure may lead to antibiotic resistance, a process that is facilitated by the relatively small genome size and rapid generation time of microbes (Burchmore, 2014).

A recent study applied comparative proteomic methodologies to identify outer membrane proteins related to kanamycin resistance in *E. coli*. Mass spectrometry and western blotting results revealed that TolC, Tsx and OstA were up-regulated, whereas MipA, OmpA, FadL and OmpW were down-regulated in kanamycin-resistant *E. coli* K-12 strain. The further evaluation of the role and expression of MipA in response to nalidixic acid, streptomycin and chloramphenicol suggested that MipA was a novel outer membrane protein related to antibiotic resistance (Li et al., 2015a).

Understanding the mechanisms by which microorganisms respond to antibiotic resistance is important not only to prolong the useful life of existing drugs, but also to discover resistance-proof drug targets. Thus, decoding the molecular weaknesses of specific strains can help us understand the molecular factors that critically influence the dissemination of mobile antibiotic resistance, the vertical spread of antibiotic resistant bacteria and the success of an infection.

Chapter 2.

Aim of the thesis

Antibiotic resistant Gram negative bacteria are responsible for life threatening human infections in nosocomial and community environments (Livermore, 2009). According to recent data, Portugal was found to be above the average European level of Gram negative antibiotic resistant isolates, such as carbapenem, cephalosporin and quinolone resistant *Escherichia coli* and *Klebsiella pneumoniae* (EARS-Net, 2015).

Currently, it is not clear how and to what extent antibiotic resistant bacteria and antibiotic resistance genes get transferred and disseminated between different microorganisms (Berendonk et al., 2015). The effect of clinically relevant antibiotic resistance genes and antibiotic resistant bacteria released from diverse anthropogenic sources, together with the abusive use of antibiotics in human and veterinary medicine, is considered to be an environmental concern with consequences in non-urban settings (Cantas et al., 2013). For this reason, there is also an urgent need to characterize areas that are affected by the influence of human activity. It is crucial to detect the emergent mobile antibiotic resistance genes and assess the pathways through which pathogenic bacteria are contaminating natural environments and making mobile antibiotic resistance available to strictly environmental bacteria (Martínez, 2012; Martínez et al., 2015). Overall, environmental and zoonotic reservoirs are yet poorly assessed with regards to clinically important bacteria and genes, and the contribution of specific rural practices remains to be studied.

The aim of this thesis was to fill the gaps mentioned above by increasing the knowledge about non-human sources of bacteria carrying mobile antibiotic resistance determinants and its impact in public health, enlightening the anthropogenic influence in the overall process.

Specific aims of the different studies included:

a) To identify the occurrence of antibiotic resistant Gram negative bacteria in environmental and zoonotic bacteria;

b) To explore the extent of mobile antibiotic resistance genes in the environment and in animals of different sources;

c) To ascertain the association of antibiotic resistance genes with mobile genetic elements of great relevance in dissemination processes;

d) To characterize at the genomic and/or proteomic level specific multidrug resistant isolates that can pose a public health risk;

This information will prove useful to determine emergent mobile antibiotic resistance genes and characterize the pathways through which resistance determinants circulate among different settings.

Chapter 3.

Assessing the molecular basis of transferable quinolone resistance in Escherichia coli and Salmonella spp. from food-producing animals and food products

This research paper was published as:

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Contributions of the authors for the following manuscript:

Daniela Jones-Dias: conception and design of study, acquisition of laboratory data, analysis of data, drafting of article and critical revision of the manuscript, final approval of manuscript;
Vera Manageiro: acquisition of laboratory data, critical revision of the manuscript, final approval of manuscript;
Ana Patrícia Francisco: acquisition of laboratory data, final approval of manuscript;
Ana Paula Martins: acquisition of epidemiological data, final approval of manuscript;
Germana Domingues: acquisition of laboratory data, final approval of manuscript;
Deolinda Louro: acquisition of laboratory data, final approval of manuscript;
Eugénia Ferreira: acquisition of laboratory data, critical revision of the manuscript, final approval of manuscript;
Manuela Caniça: conception and design of study, drafting of article and critical revision of the manuscript, final approval of manuscript.

3.1. Abstract

Enterobacteriaceae resistant to quinolones frequently arise in animals, being easily disseminated through the food chain. The aim of this study was to investigate the presence of plasmid-mediated quinolone resistance (PMQR) determinants in *Salmonella* spp. (n=183) and *Escherichia coli* (n=180) isolates, collected from food-producing animals and food products among swine, poultry, rabbits and cattle. All isolates were subjected to antimicrobial susceptibility testing and molecular screening of PMQR determinants. β-Lactamase-encoding genes, and the quinolone resistance determining region (QRDR) of *gyrA*, *gyrB*, *parC* and *parE* genes were also investigated in PMQR-positive isolates. Plasmid characterization was performed by conjugation, followed by replicon-typing. Genetic relatedness of PMQR-positive *E. coli* was examined by Multilocus Sequence Typing, while *Salmonella* was previously serotyped. The association of mobile genetic elements and PMQR was investigated through PCR mapping assays. Overall, 4.1% (15/363) isolates harboured *qnrB2* (n=3), *qnrB19* (n=3), and *qnrS1* (n=9) genes. All but one isolate presented one to four mutations in QRDR of *gyrA* or *parC* genes, which is consistent with the range of MIC values detected (0.19 to 64 mg/L) for ciprofloxacin; 60% (9/15) of *qnr*-harboring isolates were nonsusceptible to β-lactam antibiotics which was justified by the presence of β-lactamases from TEM (TEM-1, n=8; TEM-135, n=1) and SHV (SHV-108, n=1) families. Analysis of mobile genetic elements revealed that *qnr* genes were detected nearby relevant genetic elements like *intI1*, IS*Ecl2*, IS*26* and ISCR*1* and enclosed in diverse Inc type plasmids. This study illustrated the existence of Qnr-producing *E. coli* and *Salmonella* from food-producing animals, associated to specific mobile elements that might mediate their transference between species and among distinct settings.

3.2. Introduction

Fluoroquinolones are a class of antimicrobials which are effectively used in the treatment of infections in both humans and animals, being also used as prophylactic agents in food-producing animals (EMA, 2012).

Bacterial resistance to fluoroquinolones has emerged quickly and has conventionally been attributed to chromosomally encoded mechanisms that allow the alteration of quinolone targets: DNA gyrase and topoisomerase IV (Eaves et al., 2004). However, the discovery of plasmid-borne determinants has increased the genetic background on the mechanisms of quinolone resistance. Currently, there are four main PMQR mechanisms: the determinant Qnr, which includes genes such as *qnrA*, *qnrB*, *qnrS,* with several variants each, and *qnrC*

and *qnrD*, that increase resistance to both nalidixic acid and fluoroquinolones (Tran and Jacoby, 2002; Hata et al., 2005; Jacoby et al., 2006; Cavaco et al., 2009; Wang et al., 2009); the cr variant of the common aminoglycoside acetyltransferase Aac(6')-Ib, which is capable of acetylate and reduce the activity of certain fluoroquinolones (Robicsek et al., 2006b); the QepA determinant, an efflux pump that confers decreased susceptibility to hydrophilic fluoroquinolones; and the multi-resistance (MDR) efflux pump OqxAB that is also able to confer resistance to nalidixic acid and ciprofloxacin, among other antimicrobial agents (Poirel et al., 2012).

These PMQR mechanisms are frequently associated to transference events, which can be facilitated by their location on mobile genetic elements, such as transposons, insertion sequences, and integrons gene cassettes, among others, leading to the establishment of MDR (Chen et al., 2009; García-Fernández et al., 2009).

Bacteria resistant to fluoroquinolones can arise and emerge in animals, being easily transferred to humans through the food chain, which can ultimately lead to the development of infectious diseases (Poirel et al., 2012).

The lack of knowledge regarding the spread of PMQR determinants in Portugal has led us to investigate their presence among *Salmonella* spp. and *E. coli* isolated from food-producing animals and food products, and to evaluate whether they could be disseminated between different settings by mobile elements.

3.3. Materials and methods

Bacterial isolates

A total of 363 unduplicated *Salmonella* spp. (n=183) and *E. coli* (n=180) isolates were collected, during the years of 2009 and 2010, in a single food safety laboratory covering four mainland Portuguese regions (North, Center, Lisbon and Tagus Valley, and South), and sent to the National Reference Laboratory of Antimicrobial Resistances (NRL-AR) at the National Institute of Health (NIH) in Lisbon, Portugal, without any previous selection criteria. Among all isolates, 91 were collected from consumable food products which included meat (n=62), processed meat (n=22) and eggs (n=7) from three distinct animal origins: pigs (n=51), poultry (n=31), and cattle (n=9), being all *Salmonella* spp. isolates. The remaining 272 isolates (92 *Salmonella* spp. and 180 *E. coli* isolates), from poultry (n=255), pigs (n=13), rabbits (n=3), and cattle (n=1), were recovered from routine samples for bacteriological diagnosis: faecal (n=58) and environmental samples (n=4), collected using sterile boots/sock swabs, placed in sterile bags and transported to the

laboratory; macerate of organs from food-producing animals (n=191); and embryonated eggs (n=19).

Antimicrobial susceptibility testing

For all isolates, antimicrobial susceptibility testing was performed by standard disk diffusion method, and interpreted according to the Antibiogram Committee of the French Society of Microbiology (Bonnet et al., 2012), using 28 distinct antibiotics, alone or in association with β-lactamase inhibitors: nalidixic acid, norfloxacin, pefloxacin, ciprofloxacin, flumequine, marbofloxacin, enrofloxacin, amoxicillin, amoxicillin plus clavulanic acid, piperacillin plus tazobactam, cephalotine, cefuroxime, ceftriaxone, cefotaxime, ceftazidime, ceftiofur, cefepime, cefoxitine, aztreonam, imipinem, meropenem, tetracycline, kanamycin, gentamicin, chrolamphenicol, trimethoprim plus sulfamethoxazole, trimethoprim and nitrofurantoin. Minimum inhibitory concentrations (MICs) of nalidixic acid and fluoroquinolones were determined by Etest (bioMérieux, Marcy l'Etoile, France) for PMQR-producing isolates and their respective transconjugants, and interpreted according to the manufacturer's instructions. Isolates were considered MDR if they presented nonsusceptibility to three or more structurally unrelated antibiotics. Strains ATCC 25922 and CQURA270 CTX-M-15-producing *E. coli* were used as controls for both antimicrobial susceptibility testing methods.

Molecular characterization of resistance

All isolates were investigated for the presence of *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(6')-Ib-cr*, and *qepA* genes, through PCR amplification using specific primers, as previously reported (Wang et al., 2003; Park et al., 2006; Cavaco et al., 2009; Wang et al., 2009). In all PMQR-producing isolates, the QRDR of *gyrA*, *gyrB*, *parC* and *parE* genes, and class 1 and 2 integrons, were screened using primers and conditions previously reported (Lévesque et al., 1995; Everett et al., 1996; Bass et al., 1999; Leverstein-van Hall et al., 2002; Ahmed et al., 2005; Mammeri et al., 2005; Sorlozano et al., 2007). Whenever PMQR-producing isolates were nonsusceptible to penicillins, they were screened for bla_{TEM}, bla_{SHV} and bla_{OXA} genes (Manageiro et al., 2012). Positive controls were used in all PCR reactions. PCR products were purified with ExoSAP IT (USB Corporation, Cleveland, OH), and further sequenced directly, on both strands, using the automatic sequencer ABI3100 (Applied Biosystems, Warrington, UK).

The inferred amino acid sequences of QRDR-encoding genes were compared with the corresponding regions of *E. coli* K-12 (GenBank accession no. AP012306) and reference

strain LT2 (GenBank accession no. AE006468) for *E. coli* and *Salmonella* isolates, respectively.

Transfer of resistance

Transferability of the PMQR determinants was performed by broth mating-out assays using recipient strains *E. coli* C600 Rif^R, Str^R and *E. coli* J53 $NaN3^R$. Resistant *E. coli* transconjugants were then selected on MacConkey agar plates containing amoxicillin (100 mg/L) or ciprofloxacin (0.06 mg/L) plus rifampicin (250 mg/L), streptomycin (160 mg/L) or sodium azide (200 mg/L), according to the donor strain antimicrobial profile. To confirm that the transconjugants acquired the PMQR-encoding genes, we detected and identified these determinants as described previously in *Molecular characterization of resistance*.

E. coli MLST analysis

PMQR-producing *E. coli* isolates were characterized by Multilocus Sequence Typing (MLST), through the detection and identification of internal fragments from the following seven house-keeping genes: *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*. The resulting sequences were then analyzed using the *Bionumerics* version 3.5 software (Applied Maths, Sint-Martens-Latem, Belgium), assigned to respective sequence types (STs), clonal complexes (CC) and singletons, according to the *E. coli* population structure through the tools available on the *E. coli* MLST website (http://mlst.ucc.ie/mlst/dbs/Ecoli).

Plasmid analysis and characterization of the genetic environment

Plasmids obtained from both parental and transconjugant strains were assigned to incompatibility groups by PCR-based replicon typing (PBRT), using previously described conditions (Carattoli et al., 2005). Strains harboring the various Inc plasmids, belonging to the NRL-AR collection, were used as positive controls (Ferreira et al., 1992). The genetic organization of 3 *qnrB2*, 3 *qnrB19*, and 6 *qrnS1* genes was investigated by PCR mapping assays, followed by primer walking and sequencing, using different primers (Table 3.1).

Table 3.1 Primers used in the molecular characterization of PMQR determinants, β-lactamases, integrons and genetic context of Qnr-encoding genes.

Gene (s)	Forward Primer Sequence	Reverse Primer Sequence	PCR product (bp) / method[a]
qepA	GAACCGATGACGAAGCACAG	CGTCGTTAAAGCATTCTTGTCC	1013/ PCR + Seq.
qnrB	ATGACGCCATTACTGTATAA	CTAACCAATCACCGCGATGC	681/ PCR + Seq.
qnrS	ATGGAAACCTACAATCATAC	GTCAGGATAAACAACAATAC	654/ PCR + Seq.
IntI2	CTTACCTGCACTGGATTAAG	TTGCGAGTATCCATAACCTG	289/ PCR + Seq
sul1	ATGGTGACGGTGTTCGG	CTAGGCATGATCTAACCCTCG	840 / PCR + Seq.
orf513	GCCAGGTCTTGAGTATCGTC	CATGTAATTGAGTCAGCGTATC	363 / PCR + Seq.
IS*26 -qnrB*	GCCTTACATTTCAAAAACTCTG	CTAACCAATCACCGCGATGC	Variable/ PCR + Seq.
strB-qnrB	AGGTGCGACAGACGTCATAC	CTAACCAATCACCGCGATGC	Variable/ PCR + Seq.
aphaA1-qnrB	GCCCATTTATACCCATATAAAT	ATGACGCCATTACTGTATAA	Variable/ PCR + Seq.
tn3-bla$_{TEM}$	CGGTATCAGTGCCAGTTTGT	TGGGTGAGCAAAAACAGGAA	Variable/ PCR + Seq.
tn3-qnrS	CACGGACATATCAATATGCTC	GTCAGGATAAACAACAATAC	Variable/ PCR + Seq.
hip-qnrS	ATGGAAACCTACAATCATA	ACCAGATGAACAACCAGTAG	Variable/ PCR + Seq.

[a]PCR + Seq, PCR and sequencing.

3.4. Results

Susceptibility to quinolones and other antibiotic classes

Global antibiotic susceptibility values are summarized in Table 3.2 for *E. coli* and *Salmonella* spp. isolates. Regarding quinolones, we noticed a significant number of both *E. coli* and *Salmonella* spp. isolates nonsusceptible to nalidixic acid (74% and 36%) and also to fluoroquinolones, like norfloxacin (71% and 23%), pefloxacin (54% and 15%), ciprofloxacin (53% and 7%), flumequin (73% and 27%), marbofloxacin (38% and 1%) and enrofloxacin (50% and 17%), respectively.

Although nonsusceptibility values were globally higher for *E. coli* than for *Salmonella* spp. isolates, this was particularly evident for β-lactam antibiotics such as amoxicillin (74% and 35%), amoxicillin plus clavulanic acid (36% and 11%), cephalotine (27% and 0%), ceftiofur (8% and 1%) and aztreonam (9% and 1%), respectively. Nitrofurantoin was the only antibiotic for which the *Salmonella* nonsusceptibility values (47%) were higher than *E. coli* (29%). Overall, 55.1% (200/363) isolates were MDR, among which *E. coli* contributed with 36.1% (131/363).

Table 3.2. Antibiotic susceptibility of *Salmonella* spp. (n=183) and *E. coli* (n=180) isolates.

Antibiotics	***Salmonella* spp. (n=183)**				***E. coli* (n=180)**			
	Nonsusceptible		**Susceptible**		**Nonsusceptible**		**Susceptible**	
	Nº	**%**	**Nº**	**%**	**Nº**	**%**	**Nº**	**%**
Nalidixic acid	66	36	117	64	133	74	47	26
Norfloxacin	42	23	141	77	127	71	53	29
Pefloxacin	28	15	155	85	98	54	82	46
Ciprofloxacin	12	7	171	93	95	53	85	47
Flumequine	50	27	133	73	132	73	48	27
Marbofloxacin	1	1	182	99	69	38	111	62
Enrofloxacin	32	17	151	83	90	50	90	50
Amoxicillin	64	35	119	65	133	74	47	26
Amoxicillin + CLA	21	11	162	89	64	36	116	64
Piperacillin + TAZ	8	4	175	96	8	4	172	96
Cephalotine	0	0	183	100	49	27	131	73
Cefuroxime	0	0	183	100	17	9	163	91
Ceftriaxone	0	0	183	100	17	9	163	91
Cefotaxime	0	0	183	100	18	10	162	90
Ceftazidime	12	7	171	93	21	12	159	88
Ceftiofur	1	1	182	99	14	8	166	92
Cefepime	0	0	183	100	8	4	172	96
Cefoxitine	1	1	182	99	6	3	174	97
Aztreonam	2	1	181	99	17	9	163	91
Imipenem	0	0	183	100	0	0	180	100
Meropenem	0	0	183	100	0	0	180	100
Tetracycline	104	57	79	43	146	81	34	19
Kanamycin	8	4	175	96	17	9	163	91
Gentamicin	5	3	178	97	29	16	151	84
Chloramphenicol	29	16	154	84	39	22	141	78
SXT	20	11	163	89	60	33	120	67
Trimethoprim	21	11	162	89	58	32	122	68
Nitrofurantoin	86	47	97	53	53	29	127	71

CLA, clavulanic acid; TAZ, tazobactam; SXT, trimethoprim/ sulfamethoxazole.

QRDR mutations and antibiotic susceptibility of PMQR-producing isolates

Among all isolates, 4.1% (15/363) harboured PMQR genes from *qnr* family (*qrnB* and *qnrS* variants): three *qnrB2* were detected in *Salmonella* Havana from embryonated eggs, three *qrnB19* were detected in *S.* Havana from poultry, in *Salmonella* Mbandaka also from poultry and in *E. coli* from a pig, and nine *qnrS1* in *E. coli* recovered from poultry (Table 3.3). All *qnr* determinants were detected in samples gathered for diagnostic purposes. The *qnrA*, *qnrC*, *qnrD*, *aac(6')-Ib-cr* and *qepA* genes were not detected in any of the investigated strains.

Table 3.3. Characteristics of Qnr-producing isolates recovered from food-producing animals in Portugal.

Isolate[a]	Animal	MLST or Serotype[b]	PMQR	QRDR mutations[c]		MIC (mg/L)		Resistance profile[d]	β-lactamases	C1Int/ C2Int	PBRT
				gyrA	*parC*	NAL	CIP				
E. coli											
INSLA33	Pig	2298 (CC23)	QnrB19	S83L	-	>256	2	P; Q; TE	TEM-1B (P3)	+/-	N, FIB
INSLA37	Chicken	23 (CC23)	QnrS1	S83L	-	>256	0.5	P; Q	TEM-1B (P3)	-/-	-
INSLA40	Chicken	540 (singleton)	QnrS1	S83L	-	>256	0.25	P; I; C1G; C2G; C3G; M; Q	TEM-135 (P3);SHV-108	-/-	I1-Iγ, FIB
INSLA51	Turkey	23 (CC23)	QnrS1	S83L	-	>256	1	Q; TE; S	NA	+/-	N, FIB
INSLA52	Layer	156 (singleton)	QnrS1	S83L	-	>256	0.75	P; Q; TE; S	TEM-1B (P3)	-/-	FIB, FIC
INSLA73	Layer	23 (CC23)	QnrS1	S83A	-	64	0.5	P; Q	TEM-1B (P3)	-/-	FIB, FIC
INSLA 83	Broiler	2299 (CC95)	QnrS1	S83L	-	>256	1	P; Q; TE	TEM-1B (P3)	-/-	FIB, FIC
INSLA100	Broiler	156 (singleton)	QnrS1	S83L/D87N	S80I/E84G	>256	64	P; Q; TE; S	TEM-1B (P3)	+/-	FIB
INSLA240	Broiler	155 (singleton)	QnrS1	-	-	8	0.19	P; Q; TE	TEM-1B (P3)	-/-	Y, FIB
INSLA310	Broiler	140 (CC95)	QnrS1	S83L	-	>256	1	P; Q	TEM-1B (P3)	-/-	FIB, FIC
Salmonella spp.											
INSLA142	Poultry	Havana	QnrB2	D200N	-	32	0.25	Q	NA	+/-	H2, L/M
INSLA143	Poultry	Havana	QnrB2	D200N	-	16	0.25	Q	NA	+/-	H2, L/M
INSLA144	Poultry	Havana	QnrB2	D200N	-	16	0.19	Q	NA	+/-	H2, L/M
INSLA343	Poultry	Havana	QnrB19	D200N	-	16	0.5	Q	NA	-/-	H2
INSLA350	Poultry	Mbandaka	QnrB19	D200N	-	32	0.25	Q	NA	-/-	H2

P, penicillins; I, combinations of β-lactam antibiotic plus β-lactamase inhibitor; C1G, first generation cephalosporins; C2G, second generation cephalosporins; C3G, third generation cephalosporins; M, monobactam; Q, quinolones; TE, tetracycline; S, sulphonamides; NAL, nalidixic acid; CIP, ciprofloxacin; NA,non applicable, because showed susceptibility to β-lactam antibiotics

[a] Isolates that successfully transferred their PMQR determinants to recipient strains are underlined, as well as the genotypic characteristics that were maintained in transconjugants (PMQR, β-lactamases, class 1 and 2 integrons and Inc plasmids);

[b] MLST for *E. coli* and serotypes for *Salmonella* spp.;

[c] None of the isolates presented mutations in *gyrB* and *parE* genes;

[d] Antibiotic susceptibility evaluated by disk diffusion method;

PMQR, plasmid-mediated quinolone resistance; QRDR, quinolone resistance determining region; MIC, Minimum inhibitory concentration; C1Int/C2Int, class 1 and class 2 Integrons; PBRT, PCR-based replicon typing.

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Overall, 60% (9/15) of Qnr-producing isolates (QnrB19 and QnrS1) were nonsusceptible to β-lactam antibiotics, justified by the presence of β-lactamases from TEM (TEM-1, n=8; and TEM-135, n=1) and SHV (SHV-108, n=1) families (Table 3.3), and 40% (6/15) were MDR.

Eight of those *E. coli* isolates had a single mutation in codon 83 of *gyrA*: S83L (for six *qnrS1*- and one *qnrB19*-producing isolates) and S83A (for one *qnrS1*-producing isolate). Five *Salmonella* isolates presented also a single D200N substitution in *gyrA* gene (*qnrB*-producing isolates). One other *E. coli* isolate (with *qnrS1* gene) presented double mutations in *gyrA* (S83L and D87N) and *parC* gene (S80I and E84G); isolate *E. coli* LA240 presented wild-type sequences for all four QRDR investigated genes, and all the remaining isolates were confirmed to have wild-type sequences regarding *gyrB* and *parE* genes.

All Qnr-producing isolates were nonsusceptible to nalidixic acid through the disk diffusion test although three isolates were categorized as susceptible for this antibiotic by the MIC method (Figure 3.1). For ciprofloxacin, using the MIC method, thirteen of the Qnr-producing isolates were classified as susceptible, for disk diffusion only five were placed in that category (Figure 3.1).

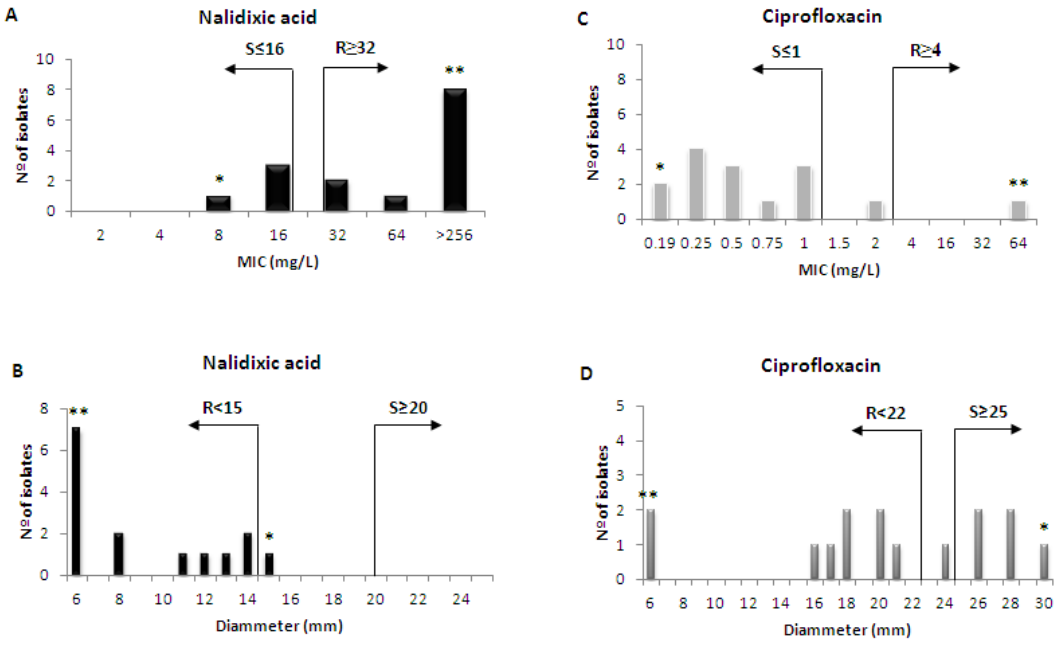

Figure 3.1. Distribution of 15 Qnr-producing isolates by susceptibility against nalidixic acid (A and B) and ciprofloxacin (C and D). MIC values (mg/L) (A and C) and inhibition zone diameters (mm) (B and D) obtained through Etest and disk diffusion test. Resistance and susceptibility categories are represented by arrows. INSLA240 (*): none mutation in QRDR; INSLA100 (**): double mutations in QRDR; all the remaining isolates harboured a single mutation in QRDR.

Diversity of Qnr-harboring *E. coli* isolates

The epidemiology and diversity of *E. coli* isolates was evaluated by MLST analysis, revealing that among the 10 Qnr-producing *E. coli* isolates, there were seven different STs, grouped in two clonal complexes (CC23 and CC95) and three singletons. Isolate *E. coli* INSLA33 (*qnrB19*) and *E. coli* INSLA83 (*qnrS1*) were assigned a new *icd* allele number and a new allele combination, which were registered in the MLST database, becoming ST2298 and ST2299, respectively (Table 3.3).

Transfer of resistance

Five isolates with *qnrS1* and three with *qnrB2* transferred their resistance into isogenic receptor strains (Table 3.3 and 3.4). The quinolone MICs of the eight transconjugants obtained, ranged from 6 to 12 mg/L for nalidix acid, 0.094 to 0.38 mg/L for ciprofloxacin, 0.5 to 1mg/L for moxifloxacin, 0.25 to 0.5 mg/L for gatifloxacin, 0.38 to 1.5 mg/L for enrofloxacin, 0.125 to 0.5 mg/L for levofloxacin, 0.38 to 1.5 mg/L for ofloxacin, and 0.25 to 1.5 mg/L for norfloxacin, revealing increases of 12- to 48-fold over that of the recipient strain (Table 3.4).

Table 3.4. MICs of antibiotics for veterinary isolates and *E. coli* transconjugants and recipients[a]

Strain[a]	MIC (mg/L) of antibiotic							
	NAL	CIP	MOX	GAT	EFX	LVX	OFX	NOR
K12 C600	1	0.008	0.032	0.023	0.032	0.012	0.032	0.047
INSLA51 (QnrS1)[b]	>256	1	3	2	12	2	6	1.5
K12 C600-LA51 (QnrS1)	6	0.125	0.75	0.25	0.5	0.125	0.5	0.5
INSLA52 (QnrS1+TEM-1)[b]	>256	0.75	2	2	4	1	3	1
K12 C600-LA52 (QnrS1+TEM-1)	8	0.38	0.75	0.38	1.5	0.38	1	1.5
INSLA83 (QnrS1+TEM-1)[b]	>256	1	2	1.5	3	1	3	1
K12 C600-LA83 (QnrS1+TEM-1)	12	0.38	0.75	0.5	1.5	0.5	1.5	1.5
INSLA142 (QnrB2)[c]	32	0.25	1	0.38	2	0.38	1	0.75
K12 C600-LA142 (QnrB2)	12	0.125	0.5	0.25	0.38	0.125	0.38	0.25
INSLA143 (QnrB2)[c]	16	0.25	1	0.38	2	0.38	1	1
K12 C600-LA143 (QnrB2)	12	0.094	0.5	0.25	0.38	0.125	0.38	0.25
INSLA144 (QnrB2)[c]	16	0.19	1	0.38	2	0.25	1	0.75
K12 C600-LA144 (QnrB2)	12	0.125	0.5	0.25	0.38	0.125	0.38	0.25
INSLA240 (QnrS1+TEM-1)[b]	8	0.19	0.75	0.38	1.5	0.38	0.75	0.75
K12 C600-LA240 (QnrS1+TEM-1)	8	0.38	0.75	0.38	1.5	0.38	1	1.5
INSLA310 (QnrS1+TEM-1)[b]	>256	1	2	1.5	3	1	3	1
K12 C600-LA310 (QnrS1+TEM-1)	8	0.38	1	0.38	1.5	0.5	1.5	1

[a] *E. coli* K12 C600 was the recipient strain. All remaining K12 C600 represent the transconjugants of the veterinary isolates immediately above; resistance determinants are referred between parentheses.
[b] *E. coli*
[c] *Salmonella* spp.
NAL, nalidixic acid; CIP, ciprofloxacin; MOX, moxifloxacin; GAT, gatifloxacin; EFX, enrofloxacin; LVX, levofloxacin; OFX, ofloxacin; NOR, norfloxacin.

Plasmid characterization and integron content

All but one *qnr*-positive isolates were positively typed by the PBRT method (Table 3.3). Positive isolates for *qrnS1* gene revealed diversity of replicon-types, varying among IncN (n=1), IncI1-Iγ (n=1), IncFIB (n=8), IncFIC (n=4) and IncY (n=1) (Table 3.3). One of the *qnrB19*-producing isolates showed the presence of types IncN and IncFIB and both the other two remaining isolates revealed to be positive to IncHI2 plasmids. All three *qnrB2* positive isolates showed the presence of IncHI2 and IncL/M plasmids. Regarding transconjugants, *qnrS1* from isolate *E. coli* INSLA51 was located in an IncN plasmid and all *qnrB2* in IncL/M plasmids. The remaining *qnrS1* positive transconjugants were negative to the 18 replicons accessed by this method (Table 3.3). Among all *qnr*-producing isolates, three *E. coli* and three *Salmonella* isolates were positive for class 1 integrons, specifically one *qnrB19*, two *qnrS1* and three *qnrB2* genes. No class 2 integrons were detected in these isolates (Table 3.3).

Genetic elements in the vicinity of PMQR determinants

Surrounding regions of *qnr* genes (Figure 3.2A) revealed that all three *qnrB2* genes were inserted in a complex integron backbone structure and were associated to an IS*CR1* element. Six out of the nine *qnrS1* positive isolates, were flanked upstream by the truncated version of IS*Ecl2*. In five isolates (*E. coli* INSLA52, INSLA73, INSLA83, INSLA240 and INSLA310) we were able to determine the nucleotide sequence between the IS*Ecl2*-*qnrS1* and the Tn*3* containing the $bla_{\text{TEM-1}}$ gene located upstream (Figure 3.2B). In addition, *Δres-orf259* region was found to be located downstream of *qnrS1* in the same isolates. Analysis of *qnrS1* region of the isolate *E. coli* INSLA51 revealed a different genetic environment from those above mentioned (Figure 3.2C). This gene was also flanked by the truncated version of the IS*Ecl2*, but instead of the Tn*3* we were able to detect the 5' region of a class 1 integron containing a *dfrA14* gene cassette and a complete IS*26* right upstream the ΔIS*Ecl2* element. For *qnrB19*-producing isolates it was only possible to identify the presence of IS*26* upstream the gene in *E. coli* INSLA33. No IS*26* was detected in association to *qnrB19* in *S*. Havana INSLA343 and *S.* Mbandaka INSLA350 isolates.

3.5. Discussion

Not much is known concerning the epidemiology of PMQR mechanisms in veterinarian isolates. This lack of knowledge assumes even greater importance if we take in account that the dissemination of plasmids carrying *qnr* genes can have serious impact and may

further compromise the efficacy of fluroquinolone treatments in both animals and humans.

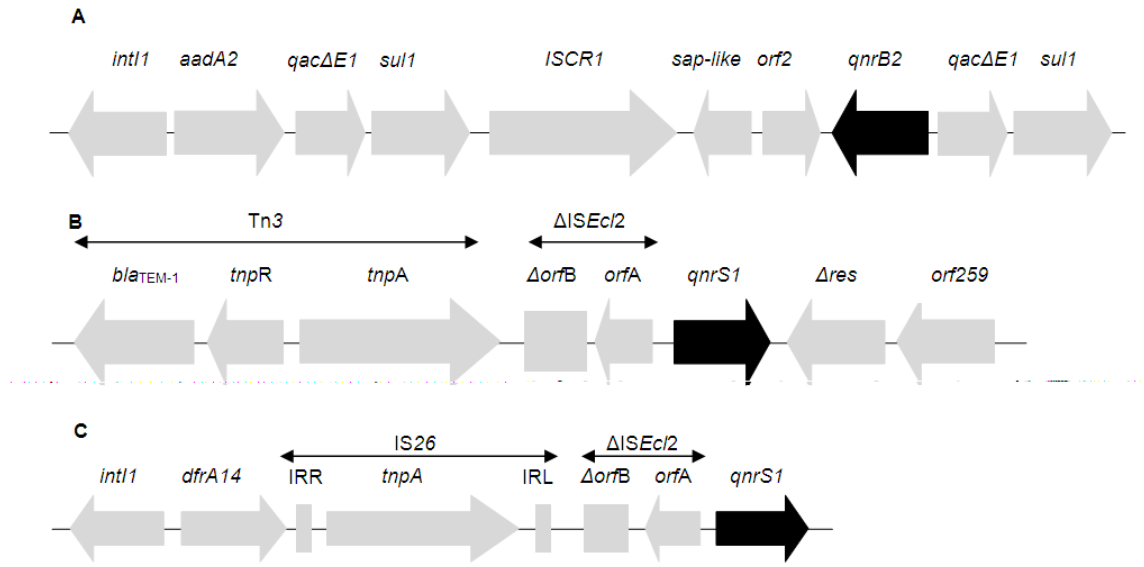

Figure 3.2. Genetic environment flanking *qnrB2* (isolates INSLA142, INSLA143 and INSLA144) (A), *qnrS1* (isolates INSLA52, INSLA73, INSLA83, INSLA240 and INSLA310) (B) and *qnrS1* genes (isolate INSLA51) (C). Arrows are drawn to scale.

In this study we evaluated the nonsusceptibility levels of *Salmonella* and *E. coli* from food-producing animals to quinolones, which revealed to be very distinct among these species. In fact, values of nonsusceptibility have leveled off between 1% (marbofloxacin) to 36% (nalidix acid) for *Salmonella* spp. and 38% (marbofloxacin) to 74% (nalidix acid) for *E. coli* isolates, revealing quinolone nonsusceptibility levels relatively high compared to other European countries (EFSA, 2010). One of the main causes of these high nonsusceptibility levels might be the usage of antimicrobials in animal husbandry, since Portugal is still a country with high antibiotic use in veterinary medicine and with a high rate of human fluoroquinolone-resistant *E. coli* when compared to other European countries (EARS-Net, 2012; EMA, 2012).

Although previous descriptions were already made for human and environmental samples in Portugal (Antunes et al., 2011; Mendonça et al., 2012), at our knowledge, we have detected for the first time the presence of PMQR-encoding genes (4.1%), among isolates from food-producing animals collected within Portugal.

In those isolates, *qnrB* (1.7%) and *qnrS* (2.5%) genes were detected (and further identified as *qnrB2*, *qnrB19* and *qnrS1*), in samples received for diagnostic purposes, and recovered from pigs and poultry. The absence of other PMQR-encoding genes may indicate that their

frequency is still low or inexistent in those reservoirs in Portugal, compared to other countries (Veldman et al., 2011). Interestingly, although QnrB determinants are normally more prevalent than QnrS in animals (Veldman et al., 2012), in this study we have detected a higher percentage of QnrS1-producing isolates. Among veterinary isolates, the higher prevalence of PMQR mechanisms is still noticed among companion and zoo animals from Eastern countries such as China, where reported rates can reach up to 66.7% (Ma et al., 2009).

Even though PMQR has been studied and reported increasingly, QRDR mutations seem to be the main quinolone resistance mechanism in animal isolates (Hordijk et al., 2011b). This is consistent with our observations: although a rate of 4.1% of PMQR was detected, the global level of fluoroquinolone nonsusceptibility reached up to 50.4%, which is probably mainly due to QRDR chromosomal mutations. The substitutions S83L and D200N in GyrA subunit were the most frequently detected in Qnr-producing isolates in *E. coli* and *Salmonella* spp., respectively, as others had already reported (Poeta et al., 2009). In fact, all but one *qnr*-producing isolate harboured, at least, one amino acid substitution in QRDR associated proteins, also highlighting the existence and importance of combined quinolone resistance mechanisms. Moreover, even though the majority of Qnr-producing isolates presented both chromosomal and plasmid-mediated quinolone resistance together, both phenotypic methods still failed to detect them (Figure 3.1), revealing the importance of PMQR surveillance even in fluoroquinolone susceptible isolates.

As frequently reported (Poirel et al., 2012), 40% of isolates with PMQR mechanisms were MDR, encoding resistance to quinolones, β-lactams, tetracycline and/or sulphonamides. Nevertheless, Qnr-producing isolates exposed in Table 3.3 showed diversity in the epidemiology of resistant plasmids and isolates; *qnrS1* gene was, in fact, associated to IncN in *E. coli* INSLA51, but no replicon-type was detected in the remaining *qnrS1*-harboring transconjugants; indeed, it is possible that these *qnrS1* genes are enclosed in other plasmids not included in the replicon-typing scheme performed in this study. While none of the *qnrB19* genes were transferable, *qnrB2* were found to be carried in IncL/M plasmids, harboured by transconjugants of *Salmonella* INSLA142, INSLA143 and INSLA144 isolates; *qnrB2* had already been described in IncL/M plasmids in association to $bla_{\text{IMP-4}}$ in *Enterobacteriaceae* clinical isolates (Espedido et al., 2008).

Although previous studies track the dissemination of *qnr* genes back to a common clonal origin (Zhang et al., 2011), here, *qnr*-carrying isolates showed genetic diversity. *qnrB19* genes were found in both *S.* Havana and *S.* Mbandaka and also in *E. coli* from different animal origins, while *qnrS1* was also detected in different animals but exclusively in *E. coli*

belonging to two distinct clonal complexes and three singletons. However, *qnrB2*-harboring *S.* Havana presented similar phenotypic and genotypic characteristics.

Previous studies found *qnr* genes inserted in structures associated to mobile genetic elements that might have been responsible for their mobilization and spread in humans (García-Fernández et al., 2009). In this study, comparable structures were found regarding *qnrB2* and *qnrS1* genes, which underline the similarities between antibiotic resistance determinants found in human and in animal isolates (Chen et al., 2009). Indeed, these genes were found to be flanked by important genetic elements (IS*CR1*, *intI1*, IS*Ecl2* and Tn*3*) and resistance genes (*aadA2*, *qacΔE1*, *sul1* and $bla_{\text{TEM-1}}$) that might contribute to the successful establishment of bacteria, and to the dissemination of MDR. Regarding *qnrB19*, although IS*Ecp1*, *aphA1* and *strB* were previously reported in their vicinity (Hordijk et al., 2011a), in this study, they were not associated to this gene in any of the three *qrnB19*-harboring *E. coli* or *Salmonella* spp.; only in *E. coli* INSLA33 it was possible to identify the presence of IS*26* upstream of the gene. Among the eight transferable plasmids obtained during conjugation experiments, a *qnrS1* gene harboured by an IncN plasmid was found to be flanked upstream by a truncated version of IS*Ecl2*, followed by an intact copy of IS*26* located nearby the 5' end of a class 1 integron, containing a *dfrA14* gene cassette (Figure 3.2). Mobile genetic elements have already been described in association with *qnrS1* genes in plasmids pAh0376, pINF5 and pK245 from *Shigella flexneri*, *Salmonella* Infantis and *Klebsiella pneumoniae*, respectively (Hata et al., 2005; Chen et al., 2006; Kehrenberg et al., 2006). Moreover, the structure identified in pK245 resembles somewhat the region detected upstream of *qnrS1* in *E. coli* INSLA51. However, contrarily to pK245, this IncN plasmid was promptly transferred to a recipient isolate, where no β-lactam resistance was observed. The region containing *qnrS1* in pK245 appears to be a composite transposon flanked by two copies of IS*26*, while a total of 10 IS*26* elements were detected in that plasmid. In our isolate, although the IS*26* and respective inverted repeats are intact, the *bla* gene between this IS and *qnrS1* seems to be missing when comparing to pK245. Interestingly, the class 1 integron 5' extremity found in this study can also be localized in pk245. However, while in isolate *E. coli* INSLA51 this structure is located only two insertion sequences away from *qnrS1*, in pk245 they are 32,613bp apart from each other (Chen et al., 2006). Considering the resemblance between pk245 and the structure found in *E. coli* INSLA51, and given the number of elements with mobilizing ability present in the plasmid, a high diffusion of this *intI1*/IS*26*-bearing *qnrS1* might be ahead.

3.6. Conclusion

In this study, *E. coli* and *Salmonella* from food-producing animals and derived food products showed a high rate of nonsusceptibility to quinolones (199/363, 54.8%), being the underlying causes both chromosomal mutations in QRDR region and the presence of plasmid borne *qnrS* and *qnrB*. These genes were also usually associated to important mobile genetic elements that can mediate their transference between different DNA molecules among diverse bacterial species, and within distinct settings. Ultimately, the coming together of these circumstances might lead to the spread of these resistance mechanisms through the food chain and to the ineffectiveness of quinolones as antimicrobial agents.

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Chapter 4.

New class 2 integron In2-4 among IncI1-positive Escherichia coli isolates carrying ESBL and PMAβ genes from food animals in Portugal

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4.1. Abstract

The impact of extended-spectrum β-lactamases (ESBLs) and plasmid-mediated AmpC β-lactamases (PMAβs) of animal origin constitutes a public health concern. In this study, 179 *Escherichia coli* from food animals and products were analyzed, among which, 15 cephalosporin resistant isolates harbouring ESBL [CTX-M-1 (n=8), CTX-M-14 (n=1), SHV-12 (n=2)] or PMAβ [CMY-2 (n=5] were identified in poultry and swine, from different farms of distinct regions of Portugal. The multiple sequence-type IncI1-driven spread of ESBLs and PMAβs, flanked by widely disseminated mobile elements, was guaranteed by ST26/IncI1-harbouring $bla_{\text{SHV-12}}$, ST12/IncI1-harbouring $bla_{\text{CMY-2}}$, ST3 and ST38/IncI1-harbouring $bla_{\text{CTX-M-1}}$ and ST1/IncI1-harbouring $bla_{\text{CTX-M-14}}$. An IS*10*-disrupted In*2-4*, presenting a new *attI2* recombination site, was also detected in a SHV-12/CTX-M-1-harbouring isolate. This study highlights the fact that animals may act as persistent sources of ESBL- and PMAβ-harboring plasmids that might be transferred to humans through direct contact or via the food chain.

4.2. Introduction

The worldwide rate of infections caused by third generation cephalosporin resistant *E. coli* has increased alarmingly it the past decades. The production of ESBL and PMAβ has become widespread among different reservoirs (de Been et al., 2014). This sort of dissemination process has mostly been attributed to mobile genetic elements (MGE) that may encode resistance to different antibiotics and promote their spread among different microorganisms and settings (de Been et al., 2014). In Portugal, the detection of mechanisms of resistance in food animals had already led to important findings (Jones-Dias et al., 2013; Clemente et al., 2015). However, many studies fail to investigate the association of specific MGE to the spread of clinically important antibiotic resistance genes. Moreover, the knowledge on the design of MGE and their interaction with antibiotic resistance is vital to the understanding of the dynamics of antimicrobial resistance (Stokes and Gillings, 2011).

The aim of the present study was to investigate the importance of ESBL and PMAβ within a group of *E. coli* from food animals, and to determine the extent of their association with MGE; in addition, we expect to contribute to the architectural knowledge of a new type of class 2 integron.

4.3. Materials and methods

A collection of 179 *E. coli* isolates was gathered in a food safety laboratory covering four regions of Portugal (North, Centre, Lisbon and Tagus Valley, and South). All *E. coli* isolates were recovered from routine diagnostic samples [macerated organs (n=174) and faecal samples (n=5)], during one year, identified by automated methods and sent to the NRL-AR/HAI at the National Institute of Health in Lisbon, without any prior selection criteria. The samples were recovered from three distinct animal origins, as follows: poultry (n=164), pigs (n=13), and rabbits (n=2), and collected as reported elsewhere (Jones-Dias et al., 2013). Antimicrobial susceptibility of all isolates was performed by disk diffusion method, as previously reported (Bonnet et al., 2013; Jones-Dias et al., 2013). Isolates nonsusceptible to third generation cephalosporins were investigated for the presence of ESBL- and PMAβ-encoding genes, transferability of resistance, Multilocus Sequence-Typing (MLST), plasmid MLST, integrons, and genetic environment of interest genes, as reported elsewhere (Clemente et al., 2015). Isolates were considered multidrug resistant (MDR) if they presented nonsusceptibility against three or more structurally unrelated antibiotics (Magiorakos et al., 2011). The content of the class 2 integron from isolate INSLA289 was also assessed through PCR mapping, using a targeting sequencing approach with primers described elsewhere (Ramírez et al., 2005; Sáenz et al., 2010). The new version of integron In*2-4* was submitted to the European Nucleotide Archive with accession number LN827615.

4.4. Results and discussion

Overall, among the 179 *E. coli* isolates recovered, we detected 15 isolates nonsusceptible to third generation cephalosporins, all of them harbouring either ESBL or PMAβ (Table 4.1). $bla_{\text{CTX-M-1}}$ (n=8) was the most frequent ESBL-encoding gene, followed by $bla_{\text{SHV-12}}$ (n=2) and $bla_{\text{CTX-M-14}}$ (n=1); $bla_{\text{CMY-2}}$ (n=5) was the only PMAβ identified. More than half of the isolates were also identified as MDR, as shown in Table 4.1. The presence of the same β-lactamases (TEM-1, CTX-M-1, CMY-2) in animals from different regions points to different sources of these β-lactamases, spread across three important regions of the country (Table 4.1). This is in agreement with the high genetic diversity noticed: one clonal complex (ST88 and ST23) and eight additional singletons (Table 4.1). Thus, the spread of ESBL and PMAβ in *E. coli* from food animals in the country strongly suggests horizontal transfer driven by MGE, rather than clonal dissemination.

Table 4.1. Phenotypic and genotypic characteristics of the 15 ESBL- and PMAβ-producing *E. coli* isolates recovered from food animals[a].

Isolate	Animal	Farm[b]	Region	Antibiotic resistance profile	MLST[c]	β-lactamases	*bla* genes flaking regions	Integron class	PBRT[d]	pMLST (CC)[e]
INSLA108	Turkey	A	Center	P;C1G;C2G;C3G;M;Q;TE;FE	ST348	SHV-12	IS*26*-bla_{SHV-12}-IS*903*	1	I1+FIC+FIB	26 (CC26)
INSLA367	Layer	B	Center	P;I;C1G;C2G;C3G;M;F	ST2451	CMY-2	IS*Ecp1*-bla_{CMY-2}-*blc-sugE*	-	I1+FIC+FIB	12 (CC12)
INSLA239	Broiler	B	Center	P;I;C1G;C2G;C3G;M;F;TE	ST429	CMY-2	IS*Ecp1*-bla_{CMY-2}-*blc-sugE*	-	I1+FIC+FIB	12 (CC12)
INSLA293	Chicken	C	Center	P;I;C1G;C2G;C3G;C4G;M;Q	ST117	CTX-M-1	IS*Ecp1*-$bla_{CTX-M-1}$-*orf477*	-	I1+FIA+FIC +FIB	3 (CC3)
INSLA294	Chicken	C	Center	P;I;C1G;C2G;C3G;C4G;M;Q;TE;S	ST88	CTX-M-1	IS*Ecp1*-$bla_{CTX-M-1}$-*orf477*	1	I1+FIC+FIB	38 (CC3)
INSLA369	Broiler	D	LTV[f]	P;I;C1G;C3G;M;F;Q;TE	ST57	CMY-2; TEM-1	IS*Ecp1*-bla_{CMY-2}-*blc-sugE*	-	I1+FIC+FIB	ND
INSLA289	Broiler	E	Center	P;I;C1G;C2G;C3G;C4G;M;Q;TE;A;FT	ST57	SHV-12; CTX-M-1	IS*Ecp1*-$bla_{CTX-M-1}$-*orf477* IS*26*-bla_{SHV-12}-IS*903*	2	I1+FIB	3 (CC3)
INSLA222	Broiler	F	Center	P;C1G;C2G;C3G;C4G;Q;TE	ST135	CTX-M-14	IS*Ecp1*-$bla_{CTX-M-14}$	-	I1	1 (None)
INSLA283	Broiler	G	Madeira	P;I;C1G;C2G;C3G;C4G;M;TE;S;FT	ST117	CTX-M-1; TEM-1	IS*Ecp1*-$bla_{CTX-M-1}$-*orf477*	1	I1+FIB	3 (CC3)
INSLA311	Piglet	H	LTV[f]	P;I;C1G;C2G;C3G;Q;TE;FE;S;FT	ST23	CTX-M-1	IS*Ecp1*-$bla_{CTX-M-1}$-*orf477*	1	I1+FIB	3 (CC3)
INSLA201	Broiler	I	Center	P;I;C1G;C2G;C3G;C4G;M;TE	ST48	CTX-M-1	IS*Ecp1*-$bla_{CTX-M-1}$-*orf477*	-	I1+FIC+FIB	3 (CC3)
INSLA256	Chicken	I	Center	P;C1G;C2G;C3G;C4G;M;TE;FE	ST48	CTX-M-1	IS*Ecp1*-$bla_{CTX-M-1}$-*orf477*	-	I1	3 (CC3)
INSLA221	Broiler	J	Center	P;C1G;C2G;C3G;C4G;M;TE	ST38	CTX-M-1; TEM-1	IS*Ecp1*-$bla_{CTX-M-1}$-*orf477*	1	I1+FIB	3 (CC3)
INSLA270	Broiler	K	Center	P;I;C1G;C2G;C3G;M;F;Q;FT	ST117	CMY-2	IS*Ecp1*-bla_{CMY-2}-*blc-sugE*	-	I1+FIB	ND
INSLA290	Chicken	K	Center	P;I;C1G;C2G;C3G;M;F;Q	ST117	CMY-2	IS*Ecp1*-bla_{CMY-2}-*blc-sugE*	-	I1+FIB	NA

[a] Features that were transferred by conjugation to recipient strains, along with the bla_{SHV-12}, $bla_{CTX-M-1, -14}$ and bla_{CMY-2} genes, are underlined; [b] Farms I, J and K belonged to the same company and are located in the same county; farms B, C and F belong to the same company, but are located in different counties; [c] MLST, Multilocus Sequence-Typing; [d] PBRT, PCR based replicon-typing; [e] pMLST, Plasmid multilocus sequence-typing; [f] LTV, Lisbon and Tagus Valley region: ND, not determined due to the presence of two *ardA* genes within the same IncI1 plasmid, which prevent us from assigning a single allele; NA, not applicable because no transconjugant was obtained from INSLA290; P, penicillins (amoxicillin); I, β-lactam/β-lactamase inhibitor combinations (amoxicillin plus clavulanic acid, piperacillin plus tazobactam, cefotaxime plus clavulanic acid); C1G, 1st generation cephalosporins (cephalotin); C2G, 2nd generation cephalosporins (cefuroxime); C3G, 3rd generation cephalosporins (ceftiofur, ceftriaxone, cefotaxime, ceftazidime); C4G, 4th generation cephalosporins (cefepime); M, monobactam (aztreonam); F, cephamycines (cefoxitin); C, carbapenems (imipenem, meropenem); Q, quinolones (nalidixic acid, norfloxacin, pefloxacin, ciprofloxacin, flumequine, marbofloxacin, enrofloxacin); TE, tetracyclines (tetracycline); A, aminoglycosides (kanamycin, gentamicin); FE, phenicols (chloramphenicol); S, sulphonamides (trimethoprim, trimethoprim plus sulfamethoxazole); FT, nitrofurans (nitrofurantoin).

All the transconjugants carrying ESBL- or PMAβ-encoding genes harboured IncI1 (14/15), and three of them also carried IncFIB (3/13) plasmids (Table 4.1). However, *E. coli* isolates harboured plasmids belonging to different replicon types: IncI1 (n=15), IncFIA (n=1), IncFIB (n=13) and IncFIC (n=7) (Table 4.1). In fact, the mobilization of the detected *bla* genes was driven by five different IncI1 plasmids: ST26/IncI1-harbouring bla_{SHV-12}, ST12/IncI1-harbouring bla_{CMY-2}, ST3 and ST38/IncI1-harbouring $bla_{CTX-M-1}$ and ST1/ IncI1-harbouring $bla_{CTX-M-14}$. $bla_{CTX-M-1}$ was associated to two different plasmids from clonal complex 3, suggesting the occurrence of intra-plasmid evolution. ST12/IncI1 and ST3/IncI1 have been associated to the spread of CTX-M-1 and CMY-2 β-lactamases in animals, which represents a concern, because they might be easily disseminated to humans, water and soil (Ben Sallem et al., 2014). Globally, although the isolates carried other plasmids, IncI1 plasmids seem to be responsible for this epidemic-like spread, which has already been cause for concern in other countries (de Been et al., 2014).

The characterization of the genetic context of ESBL- and PMAβ-encoding genes showed the presence of IS*Ecp1* upstream of the nine $bla_{CTX-M-1}$ and of the five bla_{CMY-2} genes, while the two bla_{SHV-12} genes were flanked upstream by IS*26*. The downstream genetic context of the genes consisted of *orf477* in $bla_{CTX-M-1}$ genes (n=8), IS*903* in bla_{SHV-12} and a *blc-sugE* structure in bla_{CMY-2} (Table 4.1). The detection of those IS elements, despite of the plasmid background or ST, indicates that they might play an important part in the capture, expression and mobilization of bla_{ESBL} and $bla_{PMAβ}$ genes (Stokes and Gillings, 2011; de Been et al., 2014).

Furthermore, 5 isolates were positive for class 1 integrons, and the isolate INSLA289 for a class 2 integron; the array of the latter revealed a new arrangement (Table 4.1). The integron sequence resembled the classic In*2-4*, except within the recombination site where the array containing *dfrA1*, *sat2* and *aadA1y* was preceded by a complete IS*10* that resulted in the disruption of the original *attI2* and reconstruction of a second *attI2* immediately after the inserted IS*10* (Figure 4.1) (Ramírez et al., 2005; Ramírez et al., 2010).

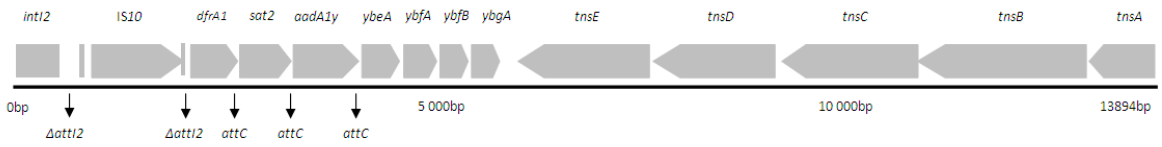

Figure 4.1. Scheme of the In*2-4* harboured by isolate INSRA289 (LN827615) where the *attI2* recombination site (793-809bp and 2,148-2,193bp) is disrupted by the insertion of a complete IS*10* (1,329bp, at position 810-2,138bp).

Consequently, integrating abilities of In*2* remained intact, and the new construct benefits of an additional capability to move using the recently acquired IS*10* transposase, which may have important consequences on resistance dissemination. Moreover, an amino acid alteration (H379Y) was detected within TnsD protein.

This study reports multiple animal sources of ST3/IncI1-harbouring $bla_{\text{CTX-M-1}}$, and other ESBL/PMAβ-encoding genes associated with the spread of epidemic plasmids within and among different farms. These genes have been increasingly indentified in humans and animals across Europe, highlighting the widespread distribution of those MGE along the food chain.

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Chapter 5.

QnrS1 and Aac(6')-Ib-cr-producing *Escherichia coli among isolates from animals of different sources: susceptibility and genomic characterization*

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Manuela Caniça: conception and design of study, critical revision of the manuscript, final approval of manuscript.

5.1. Abstract

Salmonella enterica and *Escherichia coli* can inhabit humans and animals from multiple origins. These bacteria are often associated with gastroenteritis in animals, being a frequent cause of resistant zoonotic infections. In fact, bacteria from animals can be transmitted to humans through the food chain and direct contact. In this study, we aimed to assess the antibiotic susceptibility of a collection of *S. enterica* and *E. coli* recovered from animals of different sources, performing a genomic comparison of the plasmid-mediated quinolone resistance (PMQR)-producing isolates detected.

Antibiotic susceptibility testing revealed a high number of non wild-type isolates for fluoroquinolones among *S. enterica* recovered from poultry isolates. In turn, the frequency of non-wild-type *E. coli* to nalidixic acid and ciprofloxacin was higher in food-producing animals than in companion or zoo animals.

Globally, we detected two *qnrS1* and two *aac(6')-Ib-cr* in *E. coli* isolates recovered from animals of different origins. The genomic characterization of QnrS1-producing *E. coli* showed high genomic similarity (O86:H12 and ST2297), although they have been recovered from a healthy turtle dove from a Zoo Park, and from a dog showing symptoms of infection. The *qnrS1* gene was encoded in an IncN plasmid also carrying $bla_{\text{TEM-1}}$-containing Tn*3*. Isolates harboring *aac(6')-Ib-cr* were detected in two captive bottlenose dolphins, within a time span of two years. The additional antibiotic resistance genes of the two *aac(6')-Ib-cr*-positive isolates ($bla_{\text{OXA-1}}$, $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-15}}$, *catB3, aac(3)-IIa* and *tetA*) were enclosed in IncFIA plasmids that differed in a single transposase and 60 single nucleotide variants. The isolates could be assigned to the same genetic sublineage – ST131 fimH30-Rx (O25:H4), confirming clonal spread.

PMQR-producing isolates were associated with symptomatic and asymptomatic hosts, which highlight the aptitude of *E. coli* to act as silent vehicles, allowing the accumulation of antibiotic resistance genes, mobile genetic elements and other relevant pathogenicity determinants. Continuous monitoring of health and sick animals towards the presence of PMQR should be strongly encouraged in order to restrain the clonal spread of these antibiotic resistant strains.

5.2. Introduction

Antibiotic resistance has been critically increasing over time and now constitutes one of the major health concerns worldwide. The uncontrolled use of antibiotics in human and veterinary practices, animal production and agriculture and the increasingly easiness in global transportation contributed to the dissemination of multidrug resistant pathogens that constitute a risk for humans, animals and the environment (Marshall and Levy, 2011; EFSA, 2015). Nowadays, antibiotic resistant *S. enterica* and *E. coli* are among the most problematic zoonotic bacteria, causing severe gastroenteritis in animals and humans (EFSA, 2015).

Fluoroquinolones constitute a group of broad spectrum antibiotics of critical importance, presenting applications in both human and veterinary medicines (Poirel et al., 2012). Therefore, resistance might easily emerge in animals and get transferred to humans through the food chain and direct or indirect contact. Several examples of such transmission have already been documented (Gomes-Neves et al., 2014; Damborg et al., 2015; Schmithausen et al., 2015). Fluoroquinolone resistance has emerged rapidly due to two main types of mechanisms: mutation of the chromosomal quinolone targets DNA gyrase and topoisomerase IV, and acquisition of the transferable PMQR determinants *qnr*, *qepA*, *aac(6')-Ib-cr*, and *oqxAB* (Veldman et al., 2011; Poirel et al., 2012). The alteration of chromosomal quinolone targets can lead to higher levels of resistance than PMQRs that are only able to guarantee low-level quinolone resistance. However, the ability of the latter to be spread by horizontal gene transfer constitutes a serious concern that should be addressed (Poirel et al., 2012). In fact, antibiotic resistance genes are frequently associated to mobile genetic elements such as insertion sequences (ISs), phages, transposons and plasmids, which enhance their ability to efficiently spread among different bacterial species (Stokes and Gillings, 2011). The most worrying mechanisms of resistance, which also show a transboundary spread between animals, humans and the environment, are, in fact, encoded by mobile antibiotic resistance genes. The occurrence of mobile genetic elements harboring multiple antibiotic resistance genes is also frequent, and enables the development of bacterial multidrug resistance, which may be responsible for therapeutic failures in animals or humans (Poirel et al., 2012).

In animals, as well as in humans, several factors can affect the progression and severity of an acute infection. The synchronized presence of antibiotic resistance genes, virulence factors, mobile genetic elements and other pathogenicity determinants, is ideal to the successful spread of these microorganisms in any environment (Cosentino et al., 2013).

In this study, PMQR-producing *E. coli* isolates were gathered from a collection of *S. enterica* and *E. coli* recovered from food-producing, companion and zoo animals, in the scope of their phenotypic and genotypic characterization. To further explore the genetic diversity of these isolates, as well as to understand the molecular features contributing to their spread and ability to cause infection, complete genomic sequencing was performed.

5.3. Materials and methods

Collection of bacterial isolates

This study included 89 *S. enterica* isolates recovered from breeders (n=12), broilers (n=33), layers (n=33), swine (n=6) and food products of animal origin (n=5) (Table 5.1). In poultry farms, samples were collected from feces and environment using sterile boots/sock swabs. Food products included uncooked fresh products such as minced meat, hamburgers, meat cuts, sausages and table eggs, randomly recovered at a variety of retail stores. Samples from other animal species (pigeons, partridges, ducks, pets and exotic animals) consisted of blood cultures and organs (lung, liver, spleen, kidneys and intestine) collected during post-mortem examination. All samples were examined according to ISO norm 6579:2002 applied to *Salmonella* detection in food and animal feeding stuffs. After biochemical confirmation, *Salmonella* spp. isolates were sent to the *Salmonella* National Reference Laboratory (INIAV, Lisbon) in triple sugar iron slopes or SMID plates (bioMérieux, Marcy-l'Étoile, France).

This study also included 91 *E. coli* isolates (Table 5.1) collected from food-producing animals [(bovine, swine and poultry), (n=32)], pets [(dogs, cats, horses and cage birds), (n=37)] and zoo animals [(terrestrial and aquatic mammals, birds and reptiles), (n=22)]. Samples consisted of swabs from organic fluids and cavities, fecal samples, urine samples, blood cultures and organs collected during post-mortem examination and submitted for bacteriological analysis. Suspected *E. coli* colonies obtained in MacConkey agar plates were confirmed by API 20E strips (bioMérieux, Marcy-l'Étoile, France).

Serotypes of *S. enterica*

S. enterica isolates were serotyped by the slide agglutination method using the method of Kauffmann-White scheme (Grimont and Weill, 2007).

Table 5.1. Distribution of the *S. enterica* (n = 89) and *E. coli* (n =91) isolates.

Source	*S. enterica*			*E. coli*	
	Serotypes		Total	Source	Total
	Enteritidis	Other serotypes[a]			
Breeders	12	0	12	Food	32
Layers	24	9	33	Companion	36
Broilers	32	1	33	Zoo	23
Swine	0	6	6		
Food of animal origin	3	2	5		
Total	71	18	**89**	**Total**	**91**

[a] *Salmonella* 4,5:i:- (n=1), *Salmonella* 6,7,14:-:1,2 (n=1), *Salmonella* Bradenburg (n=1), *Salmonella* Gallinarum (n=1), *Salmonella* Give (n=1), *Salmonella* Hadar (n=1), *Salmonella* Heidelberg (n=1), *Salmonella* IIIa 48:z10:- (n=1), *Salmonella* Mbandaka (n=1), *Salmonella* Rissen (n=2), *Salmonella* Typhimurium (n=3), *Salmonella* Virchow (n=4).

Antibiotic susceptibility testing

Minimum inhibitory concentrations (MICs) were determined by agar dilution following standard recommendations, using a panel of ten antimicrobial compounds: ampicillin, cefotaxime, nalidixic acid, ciprofloxacin, gentamicin, streptomycin, chloramphenicol, tetracycline, sulfamethoxazole and trimethoprim (Table 5.2). Isolates harboring PMQR determinants were further studied by determination of the MICs to a larger panel of fluoroquinolones, which included moxifloxacin, gatifloxacin, levofloxacin, ofloxacin, enrofloxacin and norfloxacin. To assess non-wild-type isolates, interpretation of results was performed according to the epidemiological cut-off values suggested by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, http://mic.eucast.org/Eucast2/). For *Salmonella* spp., the cut-off value used for sulfamethoxazole was that for sulfonamides from Clinical Standards Laboratory Institute (http://clsi.org). MIC_{50} and MIC_{90} were calculated as reported elsewhere (Schwarz et al., 2010). *E. coli* ATCC 25922 was used as the quality control strain. Isolates were considered multidrug resistant (MDR) if they presented non-wild-type phenotypes against three or more structurally unrelated antibiotics (Magiorakos et al., 2011).

Molecular characterization of resistance

All isolates were evaluated regarding the presence of *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(6')-Ib-cr*, and *qepA* genes, using primers and conditions previously described (Jones-Dias et al., 2013), and *oqxAB* genes using primers and conditions first described in this study (oqxA-F, 5'-AGAGTTCAAAGCCACGCTG-3' and oqxb-R, 5'-CTCCTGCATCGCCGTCACCA-3';

initial denaturation of 94ºC for 5 minutes; 94ºC for 30 seconds, 64ºC for 30 seconds and 72ºC for 1 minute, for 30 cycles; final step of extension of 72ºC for 5 minutes). PMQR-producing isolates were also characterized regarding the production of β-lactamase-encoding genes and conventional Multilocus sequence typing (MLST), as described elsewhere (Jones-Dias et al., 2015a).

Genomic characterization of PMQR-producing *E. coli*

The genomes of the four PMQR-producing *E. coli* (LV46221, LV46743, LV36464 and LV27950) were characterized. Genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Aarhus) and quantified using Qubit 1.0 Fluorometer (Invitrogen, Waltham). The Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA) was used to prepare sequencing libraries from 1ng of genomic DNA, according to the manufacturer's instructions. Paired-end sequencing of 150 bp reads was performed on a MiSeq (Illumina, San Diego, CA). Sequence reads were then trimmed and filtered according to quality criteria, and assembled *de novo* using CLC genomics workbench version 8.5.1 (QIAGEN, Aarhus). RAST (Rapid Annotation using Subsystem Technology) was used for subsystem annotation of the genomes (Aziz et al., 2012; Overbeek et al., 2014).

Identification of pathogenicity-related genes

Pathogenicity-related genes were detected using a variety of online web tools. PathogenFinder 1.1, ResFinder 2.1, VirulenceFinder 1.4, SerotypeFinder 1.1, MLST 1.8, pMLST 1.4 and PHAST were used to estimate the pathogenicity determinants, acquired antibiotic resistance genes, virulence factors, serotypes, MLST, plasmid MLST and phage regions, respectively, in the genomes of PMQR-producing *E. coli* (Zhou et al., 2011; Larsen et al., 2012; Zankari et al., 2012; Cosentino et al., 2013; Carattoli et al., 2014; Joensen et al., 2014; Joensen et al., 2015). ISsaga was also used to detect and annotate insertion sequences in the draft genomes of the *E. coli* isolates (Varani et al., 2011). Specific analysis of antibiotic resistance genes and respective flanking regions was carried out with *CLC genomics workbench version* 8.5.1 (Qiagen, Aarhus). Contigs carrying antibiotic resistance genes were manually assembled whenever necessary and blasted against GenBank to identify their genetic location.

Nucleotide Sequence GenBank Accession Numbers

The draft genomes of isolates LV46221, LV46743, LV36464 and LV27950 have been deposited at DDBJ/EMBL/GenBank under the accessions LRXG00000000,

LRXH00000000, LRXI00000000 and LRXJ00000000, respectively. The versions described in this paper are version LRXG01000000, LRXH01000000, LRXI01000000 and LRXJ01000000, respectively.

5.4. Results

Serotypes of *Salmonella* spp.

S. enterica serotype Enteritidis is one of the most common serotype in humans (EFSA, 2015) and it was the most frequently detected among the 89 *S. enterica* isolates (71/89, 79.8%), being present in all food animals except swine. The remaining *Salmonella* serotypes were detected in a less extent and were comprised of *Salmonella* 4,5:i:- (n=1), *Salmonella* 6,7,14:-:1,2 (n=1), *Salmonella* Bradenburg (n=1), *Salmonella* Gallinarum (n=1), *Salmonella* Give (n=1), *Salmonella* Hadar (n=1), *Salmonella* Heidelberg (n=1), *Salmonella* IIIa 48:z10:- (n=1), *Salmonella* Mbandaka (n=1), *Salmonella* Rissen (n=2), *Salmonella* Typhimurium (n=3) and *Salmonella* Virchow (n=4).

Antimicrobial susceptibility of *S. enterica* and *E. coli* isolates

Susceptibility profiles of *S. enterica* and *E. coli* isolates differed with the animal group (Table 5.2). Although high rates of non wild-type *S. enterica* were detected for nalidixic acid (from 82% to 100%) and ciprofloxacin (from 64% to 100%) in all groups, they were particularly evident in poultry, and predominant in breeders. *S. enterica* isolates recovered from other sources (swine and food products, n=11), showed higher non-wild-type phenotypes for ampicillin (36%), streptomycin (64%), tetracycline (45%), sulfamethoxazole (36%) and trimethoprim (27%) (Table 5.2). The poultry groups of breeders and broilers were mainly susceptible to ampicillin (100%), cefotaxime (100%), gentamicin (100%) and streptomycin (100%).

The frequency of non wild-type isolates was globally higher for *E. coli* than for *S. enterica* against ampicillin (minimum value of 47% versus 0%, respectively), tetracycline (minimum value of 25% versus 0%, respectively), sulfamethoxazole (minimum value of 22% versus 0%, respectively) and trimethoprim (minimum value of 19% versus 0%, respectively). Although no major discrepancies were noticed for *E. coli* in rates of non-wild-type isolates for the different animal groups, isolates recovered from food animals still presented more non-wild-type phenotypes than zoo or companion animals against nalidixic acid (41%), ciprofloxacin (41%), tetracycline (53%) and chloramphenicol (16%).

Table 5.2. MIC_{50} and MIC_{90} for *S. enterica* (n =89) and *E. coli* (n=91) isolates.

Antibiotic	*S. enterica*				*E. coli*		
	Food animals				Food animals (n=32)	Zoo Animals (n=23)	Companion Animals (n=36)
	Breeders (n=12)	Broilers (n=33)	Layers (n=33)	Others[a] (n=11)			
Na							
MIC_{50}	128	128	128	128	8	4	4
MIC_{90}	128	128	128	128	128	128	128
% Wt	0	12	6	18	59	77	78
%N-Wt	100	88	94	82	41	23	22
Cp							
MIC_{50}	0.25	0.25	0.25	0.25	0.03	0.015	0.015
MIC_{90}	0.25	0.25	0.25	0.5	8	8	8
% Wt	0	3	18	36	59	77	72
%N-Wt	100	97	82	64	41	23	28
A							
MIC_{50}	2	4	0.5	8	8	8	8
MIC_{90}	4	4	8	64	64	64	64
% Wt	100	100	94	64	53	50	53
%N-Wt	0	0	6	36	47	50	47
Ct							
MIC_{50}	0.125	0.06	0.125	0.125	≤0.06	0.06	≤0.06
MIC_{90}	0.125	0.125	0.125	2	0.125	0.125	0.125
% Wt	100	100	94	82	100	95	94
%N-Wt	0	0	6	18	0	5	6
G							
MIC_{50}	0.25	0.25	0.25	0.5	0.5	0.5	0.5
MIC_{90}	0.5	0.5	0.5	1	2	1	1
% Wt	100	100	100	100	91	91	100
%N-Wt	0	0	0	0	9	9	0
St							
MIC_{50}	2	2	4	32	4	4	4
MIC_{90}	8	4	128	64	256	256	128
% Wt	100	100	82	36	69	64	86
%N-Wt	0	0	18	64	31	36	14
T							
MIC_{50}	2	2	2	4	32	2	2
MIC_{90}	4	4	4	64	64	64	64
% Wt	92	100	91	55	47	59	75
%N-Wt	8	0	9	45	53	41	25
C							
MIC_{50}	8	4	8	8	4	8	8
MIC_{90}	8	8	8	16	64	8	16
% Wt	100	97	100	91	84	100	92
%N-Wt	0	3	0	9	16	0	8
Su							
MIC_{50}	128	128	128	128	16	32	16
MIC_{90}	128	128	128	>512	>512	>512	>512
% Wt	92	100	100	64	63	59	78
%N-Wt	8	0	0	36	38	41	22
Tp							
MIC_{50}	0.5	0.5	0.5	0.25	0.5	0.5	0.5
MIC_{90}	0.5	0.5	0.5	32	32	32	32
% Wt	92	100	100	73	81	68	81
%N-Wt	8	0	0	27	19	32	19

[a] Others, pigs (n=6) and food products of animal origin (n=5). Na, Nalidixic acid; Cp, Ciprofloxacin; A, Ampicillin; Ct, cefotaxime; G, Gentamicin; St, Streptomycin; T, Tetracycline; C, Chloramphenicol; Su, sulfamethoxazole; Tp, trimethoprim.

In *S. enterica* isolates from poultry, similar MIC_{50} and MIC_{90} values were observed for the majority of the antibiotics tested; major differences (≥3 fold dilutions) were observed for the group "others" for ampicillin (8 and 64mg/L), tetracycline (4 and 64mg/L), sulfamethoxazole (128 and >512mg/L) and trimethoprim (0.25 and 32mg/L).
For *E. coli*, the most significant differences in MIC_{50} and MIC_{90} values were observed for nalidixic acid (4 and 128mg/L), ampicillin (8 and 64 mg/L), streptomycin (4 and 128mg/L), tetracycline (2 and 64mg /L), sulfamethoxazole (16 and >512mg/L) and trimethoprim (0.5 to 32 mg/L).
While for *S. enterica* only 9.0% (8/89) MDR isolates were detected, for *E. coli*, MDR was registered in 38.9% (35/90) of the isolates, which were distributed among 16/90 isolates from food-producing animals, 9/90 isolates from companion animals, and 10/90 isolates from zoo animals.

Molecular characterization of *S. enterica* and *E. coli* isolates

Overall, among the 180 isolates studied, we have detected and identified four PMQR determinants in *E. coli* isolates: two *qnrS1* were detected in isolates recovered from a captive turtle dove (LV46221) and a pet dog (LV46743), and two *aac(6´)-Ib-cr* were isolated from *E. coli* recovered from captive bottlenose dolphins (LV36464 and LV27950). The detection of β-lactamase-encoding genes showed the presence of $bla_{\text{TEM-1}}$ in isolates LV46221 and LV46743, and $bla_{\text{TEM-1}}$, $bla_{\text{OXA-1}}$ and $bla_{\text{CTX-M-15}}$ in LV36464 and LV27950. No other PMQR- or β-lactamase-encoding genes were identified in the collection of *E. coli* and *S. enterica* isolates.

Genomic characterization of QnrS1-producing *E. coli*

The assembly of the genome sequences of the two *qnrS1*-harboring *E. coli,* LV46221 and LV46743, yielded 200 and 199 contigs (each >200 bp long), which together comprised 4,799,985 bp and 4,801,518 bp, respectively. The average coverage of LV46221 was 135.9, while LV46743 displayed 114.1 fold. The maximum contig length obtained for these genomes was 398,205 bp and 333,601 bp, respectively (Table 5.3).
The automated annotation of the draft genomes showed that LV46221 (63%, 2,879/4,618) and LV46743 (63%, 2,873/ 4,609) presented a similar number of sequences attributed to specific subsystems (Figure S5.1). General annotation of both genomes showed 109 coding sequences associated with virulence, disease and defense, as well as 143 sequences coding for functions related with mobile genetic elements, such as phages, prophages, transposable elements and plasmids. Globally, the proportion of each

subsystem was equally represented in the genomes of the two isolates (Figure S5.1). According to RAST annotation system, LV46221 and LV46743 isolates carried 77 and 86 RNAs, respectively. The bioinformatics analysis of the genetic relatedness was carried out with regard to serotype and MLST: the serotypes of both isolates were defined as O86:H12, and they also shared the assigned MLST - ST2297 (Table 5.4).

Table 5.3. Genome analysis of *E. coli* LV46221, LV46743, LV36464 and LV27950.

Isolates	LV46221	LV46743	LV36464	LV27950
Genome size (bp)	4,799,985	4,801,518	5,180,399	5,156,819
Number of contigs	200	199	136	209
Average coverage	135.9	114.1	178.7	150.1
N50 (bp)	119,356	119,356	158,975	158,977
Maximum contig (bp)	398,205	333,601	399,998	369,918
Minimum contig (bp)	208	201	486	218
Protein-coding genes	4618	4609	5107	5069
RNAs	77	86	77	76

In silico analysis of the antibiotic resistance genes (90% identity and 40% minimum length) confirmed the presence of a *qnrS1*, and identified $bla_{\text{TEM-1}}$ gene in both isolates (Table 5.4). *qnrS1* was detected in a contig with an approximate length of 11,000 bp in both cases, showing 99% of homology with a resistance region from *S. enterica* subsp. *enterica* serovar Infantis pINF5 plasmid. By mapping all contigs against this plasmid, we detected $bla_{\text{TEM-1}}$-containing T*n3*, and a disrupted IS*2*-like element upstream of *qnrS1,* as well as IS*26* transposase downstream of the gene. Other contigs showed complementary regions, revealing the presence of a fragment encoding conjugation transfer genes upstream of Tn*3* that showed homology with *Salmonella* Virchow plasmid pVQS1 (99%).

LV46221 and LV46743 showed no additional PMQR or other acquired antibiotic resistance genes. Moreover, no mutations were detected in the quinolone resistance determining region (QRDR) of genes *gyrA*, *gyrB*, *parC* and *parE,* which are known to confer high level resistance to fluoroquinolones (Veldman et al., 2011). The isolates were also characterized with regard to specific mobile genetic elements of different classes.

The screening of typable plasmids (>98% homology) enabled the identification of IncN plasmids, which were further typed as ST1 by pMLST (Table 5.4). ISsaga allowed the specialized annotation of insertion sequences and revealed a different distribution of the same elements for LV46221 and LV46743: IS*1* (3.23% and 1.96%, respectively), IS*200*_IS*605* (3.23% and 3.92%, respectively), IS*21* (3.23% and 3.92%, respectively), IS*3*

Table 5.4. General features of PMQR-harboring *E. coli* isolates recovered from animals of different sources.

Isolate	Origin	Year	Serotype	MIC (mg/L)								PMQR	Other resistance genes	Virulence factors	MLST	Plasmids	pMLST
				Mx	Cp	Ga	Le	Of	Ef	Na	Nx						
LV46221	Dove	2008	O86:H12	0.75	0.38	0.75	0.38	1	1.5	8	0.75	*qnrS1*	$bla_{\text{TEM-1}}$	*gad*, *iss*	ST2297	IncN	ST1
LV46743	Dog	2008	O86:H12	0.5	0.38	0.5	0.5	1.5	2	8	1.5	*qnrS1*	$bla_{\text{TEM-1}}$	*gad*, *iss*	ST2297	IncN	ST1
LV36464	Dolphin	2009	O25:H4	>32	>32	8	>32	>32	>32	>256	>256	*aac(6')-Ib-cr*	*aac(3)-IIa*, $bla_{\text{CTX-M-15}}$ $bla_{\text{TEM-1}}$, $bla_{\text{OXA-1}}$ *catB3*, *tetA*	*iss*, *sat*, *gad*	ST131	IncFIA, IncX	ST1
LV27950	Dolphin	2011	O25:H4	12	>32	8	8	>32	>32	>256	>256	*aac(6')-Ib-cr*	*aac(3)-IIa*, $bla_{\text{CTX-M-15}}$ $bla_{\text{TEM-1}}$, $bla_{\text{OXA-1}}$ *catB3*, *tetA*	*iss*, *sat*	ST131	IncFIA	ST1

Mx, moxifloxacin; Cp, ciprofloxacin; Ga, gatifloxacin; Le, levofloxacin; Of, ofloxacin; Ef, enrofloxacin; Na, nalidixic acid; Nx, norfloxacin.
MIC, Minimum inhibitory concentration
PMQR, Plasmid-mediated quinolone resistance
MLST, Multilocus sequence typing
pMLST, Plasmid multilocus sequence typing

(24.19% and 21.57%, respectively), IS*4* (3.23% and 3.92%, respectively), IS*481* (1.61% and 1.96%, respectively), IS*5* (1.61% and 1.96%, respectively), IS*6* (1.61% and 1.96%, respectively), IS*As1*(27.42% and 27.45%, respectively), IS*Kra4* (6.45% and 5.88%, respectively), IS*L3* (12.9% and 9.8%, respectively), ISNCY (9.68% and 11.76%, respectively) and finally Tn*3* (1.61% and 1.96%, respectively). IS*As1* was the most frequent element detected in both isolates, and IS*66* was exclusively detected in LV46743 (1.96%). Moreover, in LV46221 we identified ten prophage regions among which three were questionable but seven were intact. The latter included prophage regions reaching up to 90.6Kb, containing 133 coding sequences (Table S5.1). In turn, in LV46743, 14 different prophages were detected that included one questionable, four incomplete and nine intact phage regions; intact zones ranged between regions of 10.4Kb carrying 12 coding sequences, and 70.3Kb with 88 protein coding DNA fragments. Overall, among the prophages showing higher scores for both genomes were serotype-converting *Shigella flexnerri* bacteriophage and Enterobacteria lambda phages (Table S5.1).

The total number of pathogenicity determinants, which according with PathogenFinder includes, for instance, virulence factors, antibiotic resistance genes and mobile genetic elements, detected a similar number of sequences in the genomes of *E. coli* LV46221 and LV46743: 607 and 611 different pathogenic families showed a 93.5% certainty of the isolates being human pathogens. Finally, the virulence factor glutamate decarboxylase (*gad*) was detected in both isolates, while the increased serum survival factor *iss* was exclusively identified in LV46221 (Table 5.4).

Genomic characterization of Aac(6')-Ib-cr-producing *E. coli*

The genome sequences of isolates LV36464 and LV27950, which were known to produce Aac(6')-Ib-cr and CTX-M-15, were also compared. Their *de novo* assembly yielded 5,180,399 bp for LV36464 and 5,156,819 bp for LV27950 and displayed a mean coverage of 178.6 and 150.1 fold, respectively. Approximately, 136 and 209 contigs (each >200 bp long) were recovered for LV36464 and LV27950 with a maximum contig length of 399,998 bp and 369,918 bp, respectively (Table 5.3).

The automated annotation of the genomes showed a total number of coding sequences of 5,107 for LV36464 and 5,069 for LV27950, excluding 77 and 76 annotated RNA molecules. The distribution of the annotated coding sequences by subsystem showed an identical representation of functions in both isolates (LV36464: 368%, 3,062/5,107; LV27950: 61%, 3,081/5,069) (Figure S5.2).

The serotypes of the LV36464 and LV27950 isolates obtained upon the analysis of *fliC*, *wzy* and *wzx* genes, were defined as O25:H4. The epidemiology and diversity of *E. coli* isolates was also explored, assigning both of them to ST131 and to sublineage fimH30-Rx.

Globally, in isolates LV36464 and LV27950 seven different acquired antibiotic resistance genes were detected: *aac(6')Ib-cr, bla*$_{\text{OXA-1}}$, *bla*$_{\text{TEM-1,}}$ *bla*$_{\text{CTX-M-15}}$, *catB3, aac(3)-IIa* and *tetA*. By mapping, the main difference between the plasmids carried by these isolates was the deletion of a 2,400 bp sequence that displayed 99.7% homology with the transposase of Tn*5403*. Moreover, 60 single nucleotide variants have been detected between them. Both plasmids displayed an IncF plasmid from a ST131 *E. coli* isolate (JJ2434, unpublished) as its best blast hit. The comparative analysis with JJ2434 showed the absence of two regions of 9,329 bp and 1,740 bp that corresponded to deletions of genes coding for unknown functions, replication proteins, endonucleases, transcriptional regulators, and conjugation transfer proteins in LV36464 and LV27950 plasmids. The analysis of the QRDR of genes *gyrA* (from 67 to 106 aminoacids), *gyrB* (from 415 to 470 aminoacids), *parC* (from 47 to 133 aminoacids) and *parE* (from 450 to 528 aminoacids) revealed the presence of amino acid substitutions in *gyrA* (S83L and D87N) and *parC* (S80I and E84V) in both isolates.

A high number of mobile genetic elements was detected in the draft genomes of these isolates. Both harboured a plasmid (>98% homology) from incompatibility group IncFIA, which according to PlasmidFinder was classified as an IncFIA type 1. LV36464 accommodated an additional IncX plasmid. The distribution of insertion sequences present in LV36464 and LV27950 genomes was also globally similar: IS*1* (5.77% and 5.66%, respectively), IS*110* (3.85% and 1.89%, respectively), IS*1380* (1.92% and 1.89%, respectively), IS*200*_IS*605* (5.77% and 3.77%, respectively), IS*21* (3.85% and 3.77%, respectively), IS*3* (23.08% and 26.42%, respectively), IS*30* (1.92% and 3.77%, respectively), IS*4* (5.77% and 3.77%, respectively), IS*481* (3.85% and 3.77%, respectively), IS*6* (1.92% and 1.89%, respectively), IS*66* (11.54% and 11.32%, respectively), IS*As1* (1.92% and 1.89%, respectively), IS*L3* (19.23% and 18.87%, respectively) and IS*NCY* (9.62% and 9.43%, respectively). It is worth mentioning that the worldwide disseminated Tn*3* was only represented in the genome of LV36464 (3.39%), and IS*92* (1.89%) in LV27950. The specialized annotation of phage and prophages revealed that LV36464 harboured 17 regions: 8 intact, 6 incomplete and 4 questionable. These intact prophage regions ranged between 17.4Kb and 51.5Kb, showing different numbers of coding sequences that varied between 24 and 88. In turn, LV27950 harboured 13 prophage regions: it displayed ten intact regions spanning between 20.6 to 86.1Kb. Globally, regions from five phages were present in the genomes of both and two were exclusive of each isolate (Table S5.1).

The detection of virulence factors in the genome of LV27950 revealed the presence of an increased serum survival factor provided by an ISS-encoding gene and a secreted autotransporter toxin denominated *sat* (Table 5.4). LV36464 shared the same virulence factors and, in addition, harboured a glutamate descarboxilase-encoding gene (*gad*). The overall estimation of pathogenicity factors present in the genome of the isolates, using known proteins with recognized involvement in pathogenicity as reference, enabled us to determine that the assembled contigs of LV36464 and LV27950 matched 553 and 544 pathogenic families, which resulted in the estimation of both isolates being human pathogens (93.1% and 93.3%), confirming their zoonotic potential.

5.5. Discussion

The prevalence of antibiotic resistance genes in isolates from animal origin has been fairly assessed (Szmolka et al., 2011; Tamang et al., 2011; Bardoň et al., 2013; Clemente et al., 2015). However, taking in account the current availability of genomic characterization tools, we are now able to proceed with more detailed characterizations of these genes, in a broader context. In this study, we characterized the genome of PMQR-producing *E. coli*. To understand the antibiotic susceptibility background of these specific isolates we have also evaluated the antibiotic susceptibility phenotypes of a collection of *S. enterica* and *E. coli* recovered from animals of different origins, in which the isolates were originally included.

The levels of non-wild type phenotypes revealed to be very distinct among *S. enterica* and *E. coli*. Non wild-type isolates for fluoroquinolones were particularly evident among poultry isolates recovered from *S. enterica*. Regarding *E. coli* isolates, the frequency of non-wild-type phenotypes to nalidixic acid and ciprofloxacin was higher in food-producing animals than in companion and zoo animals, which might be due to the high consumption of veterinary antibiotics in animal industrial units, particularly tetracyclines, sulphonamides and fluoroquinolones (EFSA, 2015). Portugal still represents a European country with high antibiotic use in animals. This fact raises concerns regarding antibiotic resistance in veterinary settings (EMA, 2014). Different MIC_{50} and MIC_{90} (3-fold dilutions) were noted for some groups of each species: *E. coli* isolates for nalidixic acid, ciprofloxacin, ampicillin, streptomycin, tetracycline, sulfamethoxazole and trimethoprim, and *S. enterica* for ampicillin, cefotaxime, tetracycline and trimethoprim.

Although PMQR determinants are typically responsible by low level resistance, their presence has been increasingly reported in animals, resulting in an additional effect on the nonsusceptibility of fluoroquinolones (Ahmed and Shimamoto, 2013; Donati et al., 2014; Jamborova et al., 2015). The high MIC values of 128mg/L against nalidixic acid and 8 mg/L

against ciprofloxacin observed in some of the isolates of our collection may be associated with amino acid alterations in the QRDR. Indeed, although the fluoroquinolone nonsusceptibility is frequently compromised by target modification, the PMQR-encoding genes have the potential to spread and promote co-selection of other antibiotic resistance genes (EMA, 2014). Late reports even suggest that the spread of PMQR may not be triggered by selection pressure, which justifies the low rates of these determinants in animals, despite the high use of fluoroquinolones (Veldman et al., 2011).

Considering the high level MICs, most likely caused by QRDR chromosomal mutations that might mask the presence of PMQR, we decided in this study to retrospectively search for these determinants in all isolates of the collection, regardless of the MIC value. We have detected four PMQR-encoding genes (4/180) (two *qnrS1* and two *aac(6')-Ib-cr*) in *E. coli* LV46221, LV46743, LV36464 and LV27950 recovered from animals of different origins: a healthy turtle dove from a Zoo Park (2008), a diseased pet dog (2008), a bottlenose dolphin from a Zoo Park showing signs of respiratory infection (2009), and a second but healthy bottlenose dolphin from the same Zoo Park (2011) (Table 5.4).

The comparison of the genomes of QnrS1-producing *E. coli* revealed that isolates LV46221 and LV46743 were very similar in terms of their global pathogenicity potential, although they were recovered from animals of different classes and completely different backgrounds (Table 5.4). The absence of chromosomal mutations in the QRDR of isolates LV46221 and LV46743 corroborated the low fluoroquinolone MIC values obtained, which spanned between 0.38g/L for ciprofloxacin and 8g/L for nalidixic acid, highlighting the low level resistance conferred by QnrS1 determinants (Cavaco and Aarestrup, 2009). The plasmid region in which the *qnrS1* was enclosed in both isolates, that included the association with Tn*3*, has already been described in association with *qnrS1* genes in plasmids from *Shigella flexneri* recovered from food products, *Salmonella* Infantis from avian origin, and human clinical *Klebsiella pneumoniae* isolates, respectively (Hata et al., 2005; Chen et al., 2006; Kehrenberg et al., 2006). Moreover, we have previously detected other *qnrS1* from animals in Portugal, associated with a similar genetic environment, exclusively in food-producing animals (Jones-Dias et al., 2013). IncN plasmids harboured by LV46221 and LV46743 were assigned to ST1 by pMLST, which have also been associated with chickens and wild bird water in Czech Republic and the Netherlands, respectively (Ben Sallem et al., 2014).

Few genomic differences were noticed between the two *aac(6')-Ib-cr*- and $bla_{\text{CTX-M-15}}$-harboring *E. coli*. In fact, the isolates could be assigned to the same genetic sublineage – ST131 fimH30-Rx, confirming clonal spread. Although samples have been recovered within a reasonable time span of 2 years, their origin refers to two bottlenose dolphins of

the same species held captive in the same Zoo Park. The presence of four chromosomal alterations in the QRDR region of isolates LV36464 and LV27950 was reflected in the high levels of fluoroquinolone MICs, which ranged between 8mg/L and >256mg/L. All antibiotic resistance genes detected in LV36464 and LV27950 (*aac(6')Ib-cr, bla*$_{\text{OXA-1}}$, *bla*$_{\text{TEM-1}}$, *bla*$_{\text{CTX-M-15}}$, *catB3, aac(3)-IIa* and *tetA*) could be traced back to a single multidrug resistance IncFIA plasmid that showed 99.9% of homology with a plasmid submitted this year to Genbank in U.S.A (JJ2434, unpublished). Although 60 single nucleotide variants have been detected between the LV36464 and LV27950 plasmids, the main difference consisted of a single deletion that involved part of a transposase-encoding gene. The absence of a set of conjugation transfer proteins (*tra* genes), among other genes, highlighted the preponderance of clonal spread over horizontal gene transfer in ST131 *E. coli* (Nicolas-Chanoine et al., 2014). Although several isoforms of identical plasmids have been detected worldwide, the simultaneous resistance to β-lactams, fluoroquinolones, aminoglycosides, chloramphenicol and tetracyclines has been a permanent feature, which reinforces the advantage that it confers (Boyd et al., 2004; Zhou et al., 2015). The detection of a ST131 fimH30-Rx *E. coli* in two dolphins, which are continuously in contact with a live audience, constitutes a public health concern. These clinically relevant multidrug resistant *E. coli* isolates have been on the rise for years (Nicolas-Chanoine et al., 2014). Initially restricted to clinical contexts, recent findings suggest that their prevalence in non-clinical settings is maintained by the constant exchange of isolates throughout the time, as verified in this study (Mathers et al., 2015a).

Although *E. coli* is a common inhabitant of the gastrointestinal tract of humans and animals, the detected transposons, plasmids and bacteriophages are essential to the acquisition of pathogenicity factors that enlarge their ability to adapt to new niches, allowing bacteria to increase the capacity to cause a broad spectrum of diseases (Bien et al., 2012). All isolates displayed genomic factors that may be critical to cause a zoonotic infection and that were reflected in high probabilities for the isolates to be human pathogens (>93%). Concerning virulence factors, we detected the presence of glutamate decarboxylase, increased serum survival gene and a secreted autotransporter toxin, irregularly distributed across the four isolates (Table 5.3), which did not denote any relation with the conditions of their respective hosts. These virulence factors confer resistance to extreme acid conditions of the intestines, enable the isolate to survive complement system and cause defined damage to kidney epithelium, being indicative of their ability to cause disease (Johnson et al., 2008; Becker Saidenberg et al., 2012). Indeed, *E. coli* isolates can frequently encode a number of virulence factors, which enable the

bacteria to colonize the urinary tract and face highly effective host defenses (Bien et al., 2012).

Although fluoroquinolones are consistently used in veterinary medicine, results presented in this study indicate that PMQR determinants occurred at a low frequency in these isolates (2.2%), as previously reported (Donati et al., 2014; Jamborova et al., 2015). However, the studied groups of animals should still be considered potential reservoirs for PMQR-producing isolates, especially because there is the inherent potential for transboundary dissemination. These isolates presented a set of genetic features essential to promote their own successful spread: multiple antibiotic resistance genes carried by well known mobile genetic elements, virulence factors adequate to zoonotic transmission and numerous other pathogenicity factors.

The analysis of many bacterial genomic features showed us great genetic relatedness between the two *qnrS1*- and *aac(6')-Ib-cr*-harboring isolates. The data gathered throughout this study illustrates two scenarios: the presence of the same strain in different hosts inhabiting remote locations and the persistence of a unique strain in a single niche during a long period of time. The strains were each associated with a case of symptomatic infection (LV46743 and LV36464) and with a report of microbiological control of an asymptomatic host (LV46221 and LV27950), which reinforces the ability of *E. coli* isolates to act as silent vehicles, allowing the accumulation of antibiotic resistance determinants, mobile genetic elements and other relevant pathogenicity determinants (Mathers et al., 2015a). It is not certain whether these bacteria spread from humans to animals, between different animals or from the environment to animals. However, in the case of companion animals, but particularly zoo animals, surveillance is essential to prevent continuous dissemination. The contact between animals and owners, zookeepers, visitors and handlers raises concerns, considering that these bacteria might easily spread to humans and to other animals (Veldman et al., 2011; Ewers et al., 2012).

Permanent surveillance of health and sick animals should be strongly encouraged, regardless of their origin, in order to monitor future trends in the dissemination of resistance to fluoroquinolones and other antibiotics. Furthermore, this genome project contains valuable scientific data which will certainly be helpful for molecular epidemiological surveys of ST131 *E. coli* clonal group.

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Chapter 6.

CTX-M-15-producing Escherichia coli in dolphin, Portugal

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Teresa Albuquerque: acquisition of laboratory and epidemiological data, final approval of manuscript;
Eugénia Ferreira: acquisition of laboratory data, final approval of manuscript;
Manuela Caniça: conception and design of study, drafting of article and critical revision of the manuscript, final approval of manuscript.

**these authors contributed equally to this work.*

6.1. Abstract

The transboundary dissemination of ST131 CTX-M-15-producing *Escherichia coli* is a subject of concern. Here, we evaluated the zoonotic potential of an isolate recovered from a captive bottlenose dolphin, by comparing its genotype with the genotype of human clinical isolates, and by investigating the genetic relatedness between them. The relationship between isolates recovered from humans and animals underlines the interspecies spread of multidrug resistant ST131 *E. coli.*

6.2. Main text

The global emergence and pandemic spread of sequence type (ST) 131 CTX-M-15–producing *E. coli* among humans and its detection in livestock, companion animals, and wildlife is a major cause for concern (Carattoli, 2008; Nicolas-Chanoine et al., 2014). Hence, it is imperative to identify and explore its dissemination traits. If CTX-M-15–producing *E. coli* continues to spread among different environments, therapeutic options in veterinary and human medicine will be greatly narrowed (Carattoli, 2008). *E. coli* is one of the gram negative bacteria most frequently isolated from bottlenose dolphins (Morris et al., 2011). However, few studies about antimicrobial resistant bacteria in dolphins have been published (Greig et al., 2007; Schaefer et al., 2009; Stewart et al., 2014). We explored dissemination linkages between CTX-M-15–producing *E. coli* isolated from a marine dolphin (*Tursiops truncatus*) and clinical isolates collected during the same period from humans all over Portugal. In 2009, *E. coli* strain LV143 (previously mentioned in Chapter 5 of this Ph.D. thesis as LV36464), isolated from respiratory exudate collected through the spiracle of a female dolphin from a zoo, was sent to the National Institute for Agricultural and Veterinary Research in Lisbon, Portugal, for bacteriological and mycological analysis and antimicrobial susceptibility testing. No clinical history for the animal was available. Mycologic examination detected no fungi or yeasts. Antimicrobial susceptibility testing of the dolphin *E. coli* strain (LV143), performed by the agar dilution method and interpreted according to European Committee of Antimicrobial Susceptibility Testing (http://www.eucast.org/), revealed a non–wild-type phenotype to cefotaxime (MIC >8 μg/mL); it also showed a synergy toward clavulanic acid, suggesting production of extended-spectrum β-lactamase (ESBL).

LV143 was also non–wild-type to ampicillin (MIC >64 μg/mL), nalidixic acid (MIC >512 μg/mL), ciprofloxacin (MIC >8 μg/mL), gentamicin (MIC >32 μg/mL), and tetracycline

(MIC >64 m /mL). This isolate remained wild-type to chloramphenicol (MIC 4 μg/mL), florfenicol (MIC 8 μg/ mL), sulfamethoxazole (MIC 32 μg/mL), trimethoprim (MIC ≤0.25 μg/mL), and streptomycin (MIC 4 μg/mL). To analyze the zoonotic potential of the dolphin isolate, we selected 61 human clinical *E. coli* isolates, previously recovered from different specimens during 2004–2009 in 7 geographically separated hospitals in Portugal (Figure 6.1), from the National Reference Laboratory of Antibiotic Resistances and Healthcare Associated Infections collection. Inclusion criteria for the clinical isolates were 1) non–wild-type susceptibility to cefotaxime, 2) presumptive phenotypic ESBL production, and 3) genetic similarity by pulsed-field gel electrophoresis. Analysis of the genetic relatedness of human and dolphin isolates, determined by pulsed-field gel electrophoresis that used *Xba*I digested DNA (*7*), revealed 1 major cluster, which included 22 (35%) clinical isolates from 3 regions in Portugal and the isolate from the dolphin (Figure 6.1).

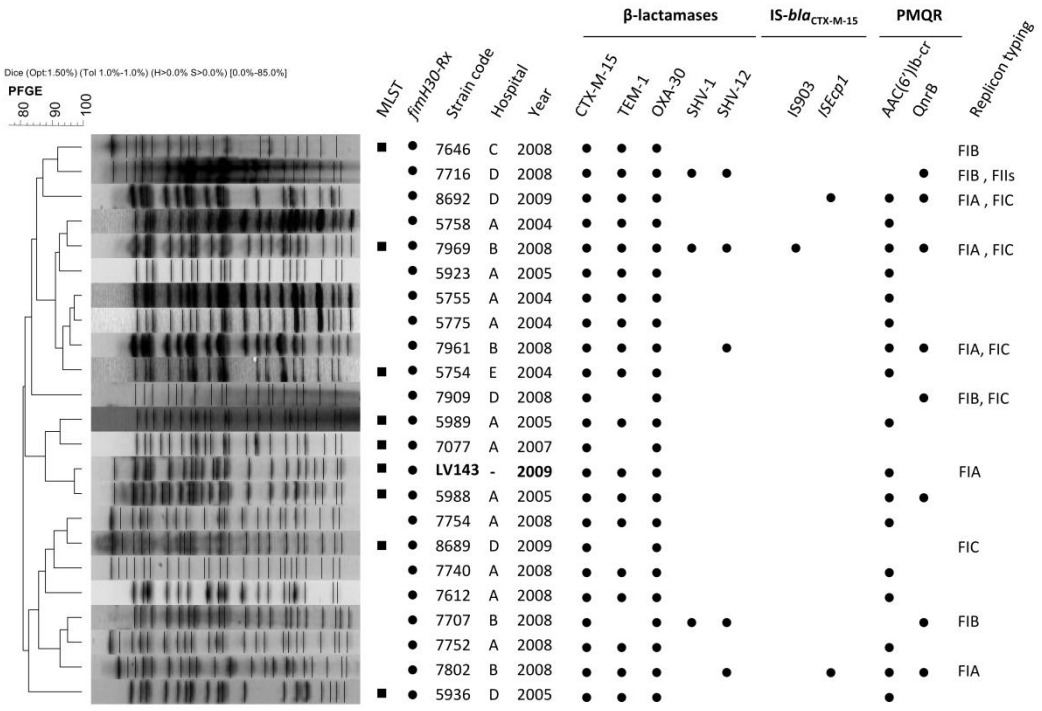

Figure 6.1. Dendogram of pulsed-field gel electrophoresis (PFGE) profiles showing the relationship between a clonal strain of *Escherichia coli* of animal origin (LV143, in boldface), and 22 *E. coli* isolates from humans. We used the unweighted pair group method and the Dice coefficient with 1.8% optimization (opt) and band position tolerance (tol) of 1%. Isolates with a Dice band–based similarity coefficient of >80% were considered to belong to the same cluster. Black squares under multilocus sequence typing (MLST) indicate sequence type (ST) 131 positivity. Year indicates year of isolation. Black circles indicate fimbral adhesin gene *fimH*, β-lactamase, IS-$bla_{\text{CTX-M-15}}$, and plasmid-mediated quinolone resistance (PMQR) positivity of indicated combinations. *E. coli* clinical isolates genetically unrelated to the dolphin isolate are not shown. Scale bar indicates percentage relatedness.

The genetic characterization of the 1 dolphin and 22 clinical isolates was performed by PCR and sequencing selective for the most prevalent ESBL-mediated genes (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA-G1}$, *bla*$_{CTX-M}$) and genes encoding plasmid-mediated quinolone resistance (*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *qepA*, *aac(6')Ib-cr*), as previously described (Manageiro et al., 2012). Specifically, the strain recovered from the dolphin contained *bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$, and *bla*$_{OXA-30,}$ associated with a plasmid-mediated quinolone resistance gene, *aac(6')-Ib-cr* (Figure 6.1). All clinical isolates were also positive for *bla*$_{CTX-M-15}$ and *bla*$_{OXA-30}$ genes; 18 isolates contained the *bla*$_{TEM-1}$ gene and 3 *bla*$_{SHV-1}$, 5 *bla*$_{SHV-12}$, 8 *qnrB*, and 16 *aac(6')-Ib-cr* genes. The presence of class 1 integrons, IS*Ecp1*, IS*26*, and IS*903* elements was also investigated, as has been done previously (Jones-Dias et al., 2015a). The LV143 strain was positive for the insertion sequence IS*Ecp1*, associated with *bla*$_{CTX-M-15}$ (Figure 6.1), and was negative for the class 1 integron (data not shown). In 2 clinical isolates, we identified IS*Ecp1*, and in 1 isolate we identified IS*903*. PCR-based replicon typing (Carattoli et al., 2005) revealed the presence of IncF plasmid group in the 1 animal and 9 human isolates (a selected sample to evaluate PCR-based replicon typing) (Figure 6.1).

Multilocus sequence typing (MLST) was performed for 9 of 23 *E. coli* isolates. According to the *E. coli* MLST website (http://mlst.ucc.ie/mlst/dbs/Ecoli), clones from the dolphin and from the humans exhibited the same combination of alleles across the 7 sequenced loci, corresponding to the epidemic ST131, associated with CTX-M-15 and widely disseminated among hospitals in Portugal (Manageiro et al., 2012; Nicolas-Chanoine et al., 2014). Within-ST subclones were analyzed on the basis of sequence variation of the *E. coli* fimbrial adhesin gene *fimH*, as previously described (Weissman et al., 2012). The *fimH30-Rx* lineage was identified in all 23 *E. coli* isolates (fluoroquinolone-resistant and CTX-M-15– positive isolates), which clustered together on the dendrogram, regardless of MLST result (Figure 6.1). It is worth noting that the *bla*$_{CTX-M}$-type gene has been detected in ESBL-positive *E. coli* isolates from healthy mammals (Carattoli, 2008). Our study illustrated clonality among clinical isolates and a dolphin strain with common antimicrobial resistance genes, specifically *bla*$_{CTX-M-15}$ and *aac(6')-Ib-cr*, and common plasmids, such as those from group IncF. These bacteria have gone through identical evolutionary genetic events, which ultimately led to the establishment of the same allelic diversity pattern (ST131 *fimH30-Rx*). The linkage between these 2 reservoirs highlights the zoonotic potential of this isolate from the dolphin.

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Chapter 7.

Influence of agricultural practice on mobile bla genes: IncI1-bearing CTX-M, SHV, CMY and TEM in Escherichia coli from intensive farming soils

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Vera Manageiro: analysis of data, final approval of manuscript;
Manuela Caniça: conception and design of study, drafting of article and critical revision of the manuscript, final approval of manuscript.

7.1. Abstract

Many calls have been made to address antibiotic resistance in an environmental perspective. With this study, we showed the widespread presence of high-level antibiotic resistant isolates, on a collection of nonsusceptible Gram negative bacteria (n=232) recovered from soils. Bacteria were selected using amoxicillin, cefotaxime and imipenem, from sites representing different agricultural practices (extensive, intensive, and organic). Striking levels of nonsusceptibility were noticed in intensive soils for norfloxacin (74%), streptomycin (50.7%) and tetracycline (46.6%); indeed, the exposure to intensive agricultural practices constituted a risk factor for nonsusceptibility to many antibiotics, multidrug resistance and production of extended-spectrum β-lactamases (ESBL).

Analyses of nonsusceptibility, highlighted that environmental and clinical bacteria from the same species might not share the same intrinsic resistance patterns, raising concerns for therapy choices in environment-borne infections. The multiple sequence-type IncI1-driven spread of penicillinases (*bla*$_{\text{TEM-1}}$, *bla*$_{\text{TEM-135}}$), ESBL (*bla*$_{\text{SHV-12}}$ and *bla*$_{\text{CTX-M-1}}$) and plasmid-mediated AmpC β-lactamases (PMAβ) (*bla*$_{\text{CMY-2}}$), produced by isolates that share their molecular features with isolates from humans and animals, suggests contamination of agricultural soils. Globally, this work constitutes the first report of *bla*$_{\text{SHV-12}}$ and *bla*$_{\text{TEM-135}}$ in soil. This is also the first appearance of IncI1/ST28-harbouring *bla*$_{\text{CTX-M-1}}$, which should be monitored to prevent their establishment as successfully dispersed plasmids. This research may help to disclose paths of contamination by mobile antibiotic resistance determinants, and the risks for their dissemination.

7.2. Introduction

Antibiotic resistance has been critically increasing over time and today constitutes one of the major concerns in human medicine. Nowadays, instead of bacteria resistant to single classes of antibiotics, pathogens frequently harbour multidrug resistance (MDR), which constitutes a serious therapeutic challenge that often cannot be overcome. First line antibiotics are becoming outdated, leading to higher morbidity, mortality and imputing high costs on the society (Laxminarayan et al., 2013; Wright, 2013).

Antibiotic resistance mechanisms of pathogenic bacteria have been broadly studied (Livermore, 2009). However, aside from specific epidemiological studies that can guarantee a local intervention with regards to infection control, they don't bring much knowledge into the dynamics and evolution of antibiotic resistance (Perry and Wright, 2013). Recently, the efforts to struggle MDR bacteria have been mainly focused on Gram

positive bacteria, which led to the implementation of important infection control measures and the development of novel drugs. However, as the scenario of Gram positive infections slightly evolved, no new antibiotics have been developed specifically for MDR Gram negative bacteria, leading to a situation where their impact is overwhelming (Giske et al., 2008). The lack of activity of today's antibiotic pipeline against Gram negative bacteria complicated infections, as well as the absence of new antibiotics created the need to also understand the ecology, evolution and dynamics of antibiotic resistance in non clinical environments (Perry and Wright, 2013). It is crucial to focus on antibiotic resistance's primary niche: the soil ecosystem, which constitutes the first and the ultimate reservoir of antibiotic resistance genes (Nesme and Simonet, 2015). In spite of this, not all resistance mechanisms constitute a threat to the therapeutic use of antibiotics (Walsh, 2013; Walsh and Duffy, 2013).

Antibiotic resistance genes associated with mobile genetic elements such as insertion sequences, plasmids, integrons and transposons pose the greatest risk, because they can be transferred from environmental settings to human pathogens and vice-versa (Stokes and Gillings, 2011). Thus, this knowledge needs to be assessed extensively, directing this investigation to resistance mechanisms that are relevant in the clinical settings, such as the production of β-lactamases, the main antimicrobial resistance mechanism in clinical Gram negative bacteria. Together, mobile β-lactamases [ESBL, PMAβ, and class A, B (metallo β-lactamases) and D carbapenemases] compromise the use of the whole class of β-lactam antibiotics (Bush, 2010).

Although the use of antibiotics (such as flumequine, tetracycline or streptomycin) in agriculture is not approved in Portugal (EC, 2009; EC, 2011), and β-lactam antibiotics are not a drug of choice in the agricultural systems, mobile *bla* genes are still emerging, disseminating and being responsible for the most worrisome resistant infections worldwide (EC, 2009; EC 2011; Gaze et al., 2013). Moreover, there are emerging concerns on the ability of anthropogenic actions to change environmental reservoirs of resistance genes, which increase the probability of recruitment of resistance genes into clinically relevant pathogens. Apart from nature being a constant source of naturally occurring antibiotic resistance determinants, selection pressure provided by diverse anthropogenic actions has to be considered (Finley et al., 2013; Gaze et al., 2013; Duffy et al., 2014). Agricultural practices have a major impact on the selection of antibiotic resistant bacteria, as they provide a positive selective pressure either by direct application of antibiotics or by indirect exposure through manure and wastewater amendments as bio-fertilizers (Popowska et al., 2012; Berendonk et al., 2015). Our hypothesis is that the inherent characteristics in different agricultural practices may be creating chemical and biological

constrains that may influence the composition of the soil, not only regarding its microbiome, but also the content of antibiotic resistance genes and mobile genetic elements.

Thus, this work aimed to assess and characterize the soil population of culturable Gram negative bacteria associated with three different agricultural practices, namely intensive, extensive and organic, with regards to their global and intrinsic nonsusceptibility, and to evaluate the presence of β-lactamase-encoding genes, exploring their association with mobile genetic elements.

7.3. Materials and methods

Soil sampling

Nine soils samples were collected from three types of agricultural settings: (1) three samples were collected from extensive agriculture (low yield multi-crop production, use of small amounts of pesticides, chemical and organic fertilizers, in a small area of land with low economic outputs); (2) three samples from intensive agriculture (high-yield single-crop production, large inputs of pesticides, chemical and organic fertilizers, big areas of land with big economic outputs); and (3) three samples from organic agriculture (low yield multi-crop production, use of small amounts of organic fertilizers, in a small former pristine land with low economic outputs) (Table 7.1). The sampling period took place in two periods: the first replicate of samples was collected in June or July of 2012 and the second in February 2013, covering the two main seasons of summer and winter. The time-span of two seasons was decided in order to make the sampling as inclusive as possible.

A sterile metal spatula was used to collect 100-mm-long soil cores that were stored under aseptic conditions in sterile propylene flasks and transported to the laboratory where they were processed within 24h of collection.

There is no record of antibiotic use for intensive or extensive soils; organic soils did not apply any antibiotics. However, it should be considered that organic fertilizers may already contain traces of antibiotic residues. Soil description, GPS coordinates, temperature and humidity records were registered for all sampling sites (Table 7.1).

Table 7.1. Geographical, climatic and descriptive characteristics of the analyzed soil samples.

Sample ID[a]	Type of agriculture	Soil culture description	Month	Latitude	Longitude	Temperature (ºC)	Humidity (%)
SL1	Extensive	Apricot tree	June	39.57434	-8.23961	31.0	27.0
			February			14.3	63.0
SL2	Intensive	Tomato[b]	June	39.20182	-8.63721	29.8	23.0
			February			13.6	53.0
SL3	Intensive	Corn[b]	June	39.13277	-8.70083	29.0	27.0
			February			13.9	41.0
SL4	Intensive	Vineyard	June	39.13252	-8.69985	29.0	27.0
			February			14.1	40.0
SL5	Extensive	Lemon tree	June	38.56530	-8.93501	27.8	26.0
			February			13.0	70.0
SL6	Extensive	Watermelon[b]	June	38.56612	-8.93551	27.6	28.0
			February			13.1	73.0
SL7	Organic	Mixed vegetables[c]	July	38.32217	-8.56273	33.0	30.0
			February			13.1	73.0
SL8	Organic	Mixed vegetables[d]	July	38.32217	-8.56273	33.0	22.0
			February			8.1	51.0
SL9	Organic	Olive tree	July	38.32217	-8.56273	29.1	25.0
			February			7.9	51.0

[a] Each soil sample refers to two replicates collected in the summer (June/July) and in the winter (February) seasons.
[b] Soil samples recovered from tomato, corn and watermelon cultures in the winter season refer to the fallow of the summer culture;
[c] Soil samples recovered from culture of mixed vegetables comprising cabbage, onions, lettuce and chards.
[d] Soil samples recovered from culture of mixed vegetables comprising cabbage, tomatoes, chards and spinach.

Selection and identification of antibiotic resistance bacteria

The soil was processed by removing all large particles and plant materials. Then, samples were incubated in Brain Heart Infusion (BHI) enrichment broth at 35 ± 2ºC during 6h followed by selection of resistant Gram negative bacteria in MacConkey agar plates containing standard concentrations of 2mg/L of cefotaxime, 2mg/L imipenem or 100mg/L of amoxicillin. Those consist of standard concentrations to select the production of broad- and extended spectrum β-lactamases, cephalosporinases and carbapenemases (Bonnet et al., 2013). Individual colonies were then selected based on their morphology so that no duplications were included. The isolates were identified through the amplification of the 16S ribosomal RNA (rRNA) genes using bacterial 16S primers: 8F 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R 5'-GGTTACCTTGTTACGACTT-3'; for isolates that no PCR product was produced a second set of primers were used 16SF 5'-AGAGTTTGATCMTGGCTCAG-3' and 16SR 5'-GTAAGGTTCTKCGCGTTGC-3'. PCR products were then purified with ExoSAP IT (USB Corporation, Cleveland, OH), and further sequenced directly, on both strands, using automatic sequencer ABI3100 (Applied Biosystems, Warrington, UK). The resulting sequences were then analyzed using the *Bionumerics* software (Applied Maths, Sint-Martens-Latem, Belgium) and assigned to respective identification, using the tools available on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Antibiotic susceptibility testing

Antimicrobial susceptibility was performed by disk diffusion method, according to the clinical species specific and species non-specific guidelines, recommendations 2013, of the Antibiogram Committee of the French Society of Microbiology (SFM) (Bonnet et al., 2013). The clinically important Gram negative bacteria isolated (n=134), defined according with Bonnet et al. (2013), were also evaluated with regards to their intrinsic nonsusceptibility profile; as no intrinsic resistance definition is available for environmental isolates, we defined it as resistance of the majority of the bacterial population (≥80%). The following different classes of antibiotics (Biorad) were tested: β-lactams [amoxicillin (25 μg), ticarcillin (75 μg), cephalotin (30 μg), cefuroxime (30 μg), cefotaxime (30 μg), cefpodoxime (10 μg), ceftazidime (30 μg), ceftriaxone (30 μg), cefepime (30 μg), aztreonam (30 μg), cefoxitin (30 μg), ertapenem (10 μg), meropenem (10 μg), imipenem (10 μg), amoxicillin plus clavulanic acid (20+10 μg), piperacillin plus tazobactam (75+10 μg)], quinolones [nalidixic acid (30 μg), norfloxacin (5 μg), ciprofloxacin (5 μg)], aminoglycosides

[kanamycin (30 µg), gentamicin (15 µg), amikacin (30 µg), streptomycin (10 µg)], phenicols [chloramphenicol (75 µg)], tetracyclines [tetracycline (30 µg)], sulphonamides [trimethoprim plus sulfamethoxazole (1.25+23.75 µg)], and furanes [nitrofurantoin (30 µg)].

All isolates were also phenotypically tested for the presence of ESBL by comparison of cefotaxime/ceftazidime (30 µg) with cefotaxime/ceftazidime plus clavulanic acid (30+10 µg) and also for metallo-β-lactamases by comparison of imipenem (10µg) with imipenem plus EDTA (10+ 750 µg). *Escherichia coli* ATCC® 25922™ and CQURA270 CTX-15-producing *E. coli* strains were also tested as control of this technique. Isolates were considered MDR when presenting reduced susceptibility to three or more structurally unrelated antibiotics.

Statistical analysis of nonsusceptibility results

We tested for positive and negative associations between each type of agricultural practice (intensive, extensive and organic) and nonsusceptibility to different antibiotics, MDR and production of ESBL. All tested antibiotics were analysed individually, except for β-lactams, which were divided in three main groups: third generation cephalosporins (cefotaxime), fourth generation cephalosporins (cefepime), and carbapenems (ertapenem, meropenem, imipenem) (Table 7.2; only factors identified as significant are shown: $p \leq 0.05$). OpenEpi software, version 3.03a (Dean et al., 2015) was used for statistical analysis. Fisher exact test was used to assess differences in antibiotic nonsusceptibility between different groups. One-tailed p values of ≤0.05 were considered to be statistically significant. Associations were determined by calculation of odds ratios with 95% confidence intervals. The null hypothesis was rejected for p values of ≤0.05.

Antibiotic resistance genes screening and identification

All isolates were investigated for the presence of genes encoding ESBL (bla_{TEM}, bla_{SHV}, bla_{CTX-M} and bla_{OXA}), PMAβ (bla_{MOX}, bla_{CMY}, bla_{FOX}, bla_{DHA}, bla_{ACT}, bla_{ACC} and bla_{MIR}) and class A (bla_{KPC}, bla_{SME}, bla_{NMC}, bla_{GES}), B (bla_{VIM}, bla_{IMP} and bla_{NDM}) and D (bla_{OXA-48}) carbapenemases by conventional PCR, as reported elsewhere (Manageiro et al., 2013). Amplification products were then purified and sequenced, as previously described in this section. Template DNAs from the collection of the National Reference Laboratory of Antibiotic Resistances and Healthcare Associated Infections were used as positive controls in all PCR reactions.

MLST profiling of soil *E. coli* isolates

The *bla*-harbouring *E. coli* isolates were characterized by Multilocus Sequence Typing (MLST), as previously reported (Wirth et al., 2006). The resulting sequences were then analyzed with the *Bionumerics* software (Applied Maths, Sint-Martens-Latem, Belgium) and eBURST (http://eburst.mlst.net/) and assigned to respective sequence types (STs) using the tools available on the *E. coli* MLST website (http://mlst.warwick.ac.uk/mlst/).

Plasmid detection and characterization

Transferability of the *bla* genes determinants was performed by mixing equal volumes of donor and recipient strains (*E. coli* C600 resistant to rifampicin or streptomycin) on Brain Heart Infusion broth. Resistant *E. coli* transconjugants were then selected on MacConkey agar plates containing cefotaxime (2mg/L) plus rifampicin (250mg/L). Transconjugants were subjected to detection and identification of *bla* genes as described before. Then, plasmids obtained from both parental and transconjugant strains were assigned to incompatibility groups by PCR-based replicon typing (PBRT), using previously described conditions (Carattoli et al., 2005). IncI1 plasmids were further characterized by plasmid Multilocus Sequence Typing (pMLST), as previously described (García-Fernández et al., 2008). The resulting sequences were then analyzed with the *Bionumerics* software (Applied Maths, Sint-Martens-Latem, Belgium) and assigned to sequence types (STs) using the pMLST website http://pubmlst.org/plasmid.

Determination of mobile genetic elements linked with the detected *bla* genes

Genetic context of *bla* genes was investigated by PCR mapping, using primers that specifically targeted IS*Ecp1*, Tn*3,* IS*26,* IS*903*, *orf477, blc* and *sugE* regions, in combination with primers directed for bla_{TEM}, bla_{SHV}, bla_{CMY} and $bla_{CTX\text{-}M}$ genes, as described elsewhere (Eckert et al., 2006; Jones-Dias et al., 2013; Jones-Dias et al., 2014; Manageiro et al., 2015b).

7.4. Results

Characterization of the culturable Gram negative population in soil

Two hundred and thirty two Gram negative antimicrobial isolates selected based on their resistance to amoxicillin, cefotaxime and/or imipenem, were cultured from eighteen samples, two replicates from each of the recovered nine soils of extensive, intensive and

organic agricultural settings (Table 7.1).

16S rDNA profiling revealed that 99.1% of the isolates were *proteobacteria* distributed among *β*- (85.3%), *γ*- (8.2%), and *α-proteobacteria* (5.6%); the remaining 0.9% comprised unclassified bacteria. These classes were represented by seven different orders including *Pseudomonadales* (50.0%), *Enterobacteriales* (25.9%), *Burkholderiales* (8.2%), *Rizhobiales* (5.6%), *Xanthomonadales* (5.6%), *Aeromonadales* (3.4%), and *Vibrionales* (0.4%), among others with minor expression (0.9%) (Figure 7.1). The predominant genus was *Pseudomonas* spp., which was more distributed in summer (45.8%) than in winter seasons (27.9%), and more present in organic (43%), than in extensive (20%) and intensive (19%) agricultural settings (Figure 7.1, S7.1 and S7.2). However, the winter season showed a slightly higher number of resistant isolates (58.6%) (Figure S7.3), among which were registered all *E. coli*, *Aeromonas*, *Proteus* and the majority of *Acinetobacter* spp. (21.3%) isolates, while *Achromobacter* (13.5%) and *Ochrobactrum* (12.5%) were mainly detected in the summer period (Figure 7.1 and S7.1).

Evaluation of antibiotic susceptibility

The great majority of the isolates (85.6%) were MDR, among which more than 60% were resistant to 14-23 different antibiotics (Figure S7.4), mainly distributed among intensive soils (P=0.05) (Table 7.2). Globally, β-lactam nonsusceptibility ranged from 8.9% and 17.8% for imipenem to 83.5% and 95.9% for cefuroxime in isolates from extensive and intensive soil samples, respectively (Figure 7.2). Isolates from organic soil samples ranged between 17.5% for cefepime to 90.0% for both cephalotin and cefuroxime. Small variations regarding global β-lactam nonsusceptibility were detected among the different soils: an average of 59.8% for organic, 56.5% for extensive and 61.7% for intensive soil samples. Globally, values of nonsusceptibility for quinolones ranged among 3.8% for ciprofloxacin in organic soils and 74% for norfloxacin in intensive soils, displaying an average value of 32.7% (Figure 7.2). Indeed, the levels of nonsusceptibility to norfloxacin were significantly higher in intensive soils ($p \leq 0.01$) (Table 7.2). Nonsusceptibility to the four aminoglycosides tested was also quite variable. Overall, it ranged between low levels of nonsusceptibility to gentamicin and amikacin (0.0% for isolates from organic soils) to greater levels of nonsusceptibility to streptomycin (50.7% for isolates from intensive soils).

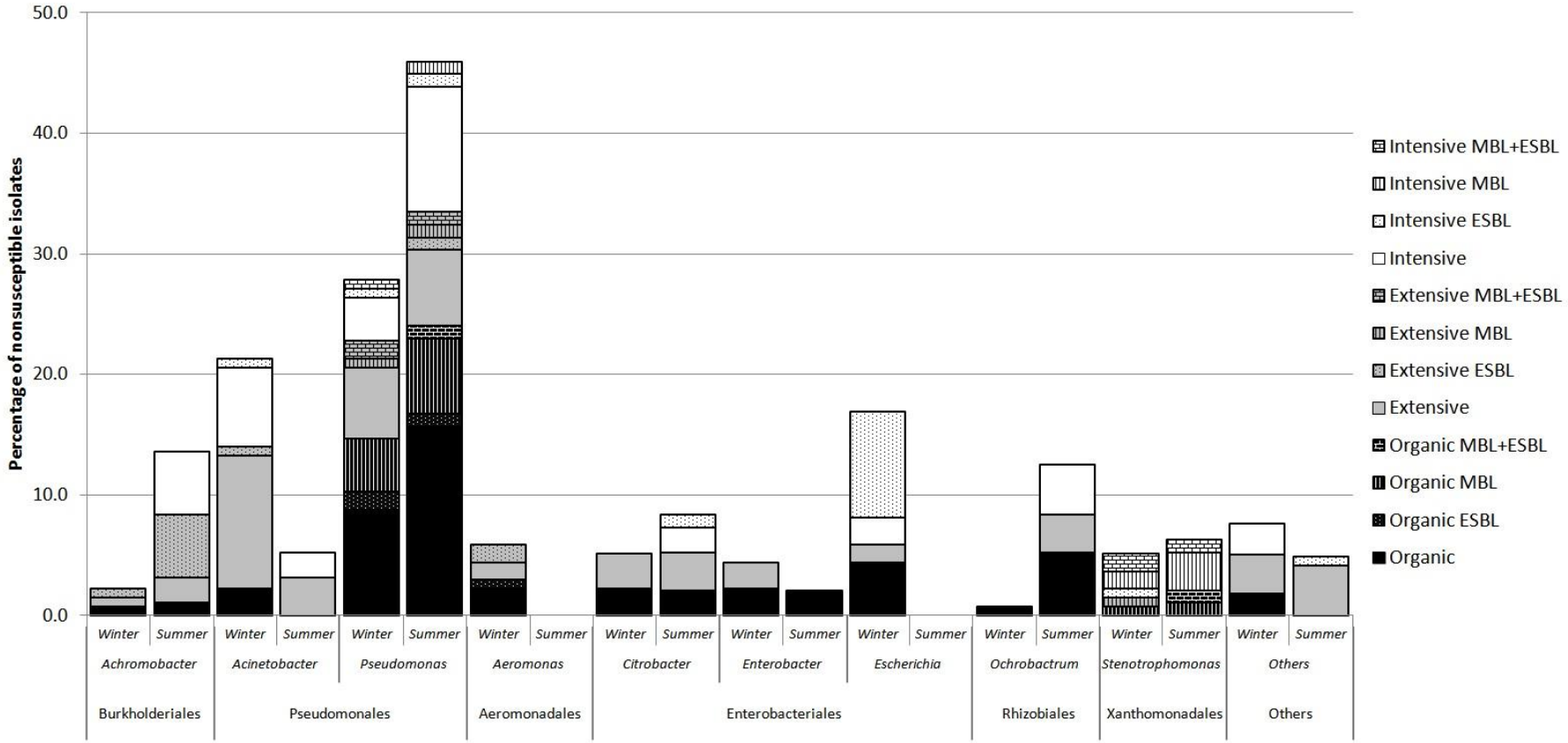

Figure 7.1. Percentage of antibiotic susceptibility of Gram negative bacteria selected as nonsusceptible to amoxicillin, cefotaxime and/or imipenem by season, type of agriculture, taxonomic group and phenotype indicative of extended-spectrum β-lactamase (ESBL) and metallo β-lactamase (MBL) production. Organic, black; Extensive, grey; Intensive, white. Dots, ESBL; Lines, MBL; Bricks, ESBL and MBL; No pattern, absence of ESBL and MBL.

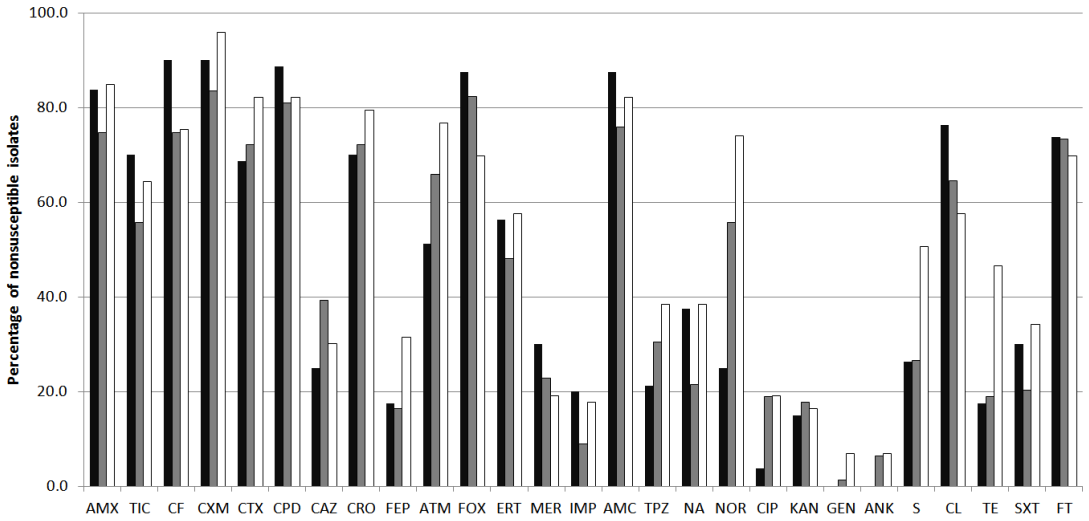

Figure 7.2. Antibiotic susceptibility profile from 232 Gram negative bacteria (previously selected as nonsusceptible to amoxicillin, cefotaxime and/or imipenem) recovered from soil, among three types of agricultural practice. Organic, black; Extensive, grey; Intensive, white.
AMX, amoxicillin; TIC, ticarcillin; CF, cephalotin; CXM, cefuroxime; CTX, cefotaxime; CPD, cefpodoxime; CAZ, ceftazidime; CRO, ceftriaxone; FEP, cefepime; ATM, aztreonam; FOX, cefoxitin; ERT, ertapenem; MER, meropenem; IMP, imipenem; AMC, amoxicillin plus clavulanic acid; TPZ, ticarcillin plus tazobactam; NA, nalidixic acid; NOR, norfloxacin; CIP, ciprofloxacin; KAN, kanamycin; GEN, gentamicin; ANK, amikacin; S, streptomycin; CL, chloramphenicol; TE, tetracycline; SXT, sulfamethoxazole; FT, nitrofurantoin.

Indeed, intensive soil samples registered a level of nonsusceptibility to streptomycin quite high (50.7%, $p \leq 0.01$) when compared to extensive (26.6%) and organic soils (26.3%) (Figure 7.2 and Table 7.2). Nonsusceptibility to chloramphenicol and tetracycline revealed opposite trends: while for tetracycline the intensive soil isolates were 29.1% more resistant than isolates from organic soils, for chloramphenicol the organic soil isolates were 18.2% more resistant than intensive soil isolates (Figure 7.1 and Table 7.2).

Table 7.2. Odds ratios (OR) and 95% confidence intervals (CI) ($p \leq 0.05$) from the analysis of negative and positive correlations for Gram negative nonsusceptible bacteria detected in intensive, extensive and organic agricultural soils.

Antibiotic	Agricultural practice	Season	OR[a]	95% CI	*p* value
Cefotaxime	Intensive	All	1.981	0.962-4.314	0.03
	Organic	Winter	0.494 (P)	0.235-1.057	0.04
Cefepime	Intensive	All	2.427	1.212-4.863	≤0.01
	Intensive	Winter	3.073	1.398-6.675	≤0.01
	Organic	Winter	0.300 (P)	0.074-0.896	≤0.01
Carbapenemes	Extensive	Summer	0.307 (P)	0.118-0.740	≤0.01
Nalidixic acid	Extensive	All	0.463 (P)	0.230-0.896	≤0.01
Norfloxacin	Intensive	All	3.941	2.091-7.655	≤0.01
	Intensive	Winter	4.692	2.061-11.76	≤0.01

	Organic	All	0.185 (P)	0.095-0.349	≤0.01
	Organic	Summer	0.281 (P)	0.110-0.658	≤0.01
	Organic	Winter	0.243 (P)	0.105-0.528	≤0.01
	Extensive	Winter	2.027	1.007-4.196	0.02
	All	Summer	0.615 (P)	0.350-1.075	0.05
	All	Winter	1.625	0.930-2.854	0.05
Ciprofloxacin	Intensive	Winter	2.735	1.081-6.643	0.02
	Organic	All	0.166 (P)	0.031-0.564	≤0.01
	Organic	Summer	0.000 (P)	0.000-0.614	≤0.01
	Extensive	Winter	3.205	1.324-7.602	≤0.01
	All	Summer	0.223 (P)	0.064-0.620	≤0.01
	All	Winter	4.483	1.613-15.52	≤0.01
Kanamycin	Organic	Winter	0.311 (P)	0.058-1.066	0.03
	All	Summer	3.313	1.521-7.530	≤0.01
	All	Winter	0.302 (P)	0.133-0.657	≤0.01
Gentamicin	Intensive	All	11.250	1.227-541.40	≤0.01
	Intensive	Winter	9.450	1.303-107.90	≤0.01
Amikacin	Organic	All	0.000 (P)	0.000-0.819	≤0.01
	Extensive	Summer	4.970	0.966-22.60	≤0.01
Streptomcyin	Intensive	All	2.749	1.488-5.112	≤0.01
	Intensive	Winter	2.871	1.382-6.024	≤0.01
	Organic	All	0.578 (P)	0.301-1.084	0.05
	Extensive	Winter	0.509 (P)	0.219-1.101	0.05
Chloramphenicol	Intensive	All	0.541 (P)	0.293-1.000	0.03
	Intensive	Winter	0.454 (P)	0.219-0.943	0.02
	Organic	All	2.031	1.070-3.975	≤0.01
	Organic	Summer	3.516	1.273-12.12	≤0.01
	All	Summer	1.970	1.073-3.686	≤0.01
	All	Winter	0.508 (P)	0.271-0.932	≤0.01
Tetracycline	Intensive	All	4.137	2.156-8.043	≤0.01
	Intensive	Winter	5.468	2.575-11.86	≤0.01
	Organic	All	0.447 (P)	0.211-0.903	≤0.01
	Organic	Winter	0.152 (P)	0.029-0.506	≤0.01
	Extensive	All	0.470 (P)	0.221-0.950	0.02
	Extensive	Winter	0.471 (P)	0.178-1.106	0.05
SXT	Extensive	All	0.554 (P)	0.270-1.093	0.05
Nitrofurantoin	Intensive	Summer	4.048	1.181-21.59	≤0.01
	Intensive	Winter	0.351 (P)	0.166-0.741	≤0.01
	Organic	Summer	3.380	1.123-13.76	≤0.01
	Extensive	Summer	2.737	0.894-11.26	0.04
	All	Summer	4.902	2.329-11.15	≤0.01
	All	Winter	0.204 (P)	0.090-0.429	≤0.01
MDR	Intensive	All	2.328	0.885-7.236	0.05
	Intensive	Winter	4.103	0.975-36.79	0.03
ESBL	Intensive	All	3.730	1.723-8.248	≤0.01
	Intensive	Winter	6.017	2.623-13.91	≤0.01
	Organic	All	0.305 (P)	0.100-0.788	≤0.01
	Organic	Winter	0.311 (P)	0.058-1.066	0.03

SXT, trimethoprim/sulphamethoxazole; MDR, Multidrug resistance; ESBL, Extended-spectrum β-lactamase.

Only significant associations are presented: *p* values ≤0.05 and confidence limits excluding null values (0, 1, or [n]).

[a] (P) indicates an OR value for a protective or negative association; otherwise, values should be interpreted as a positive association.

Moreover, 16.4% of the isolates were phenotypically positive for ESBL production by the presence of synergy between cefotaxime and clavulanic acid, ceftazidime and clavulanic acid or both, distributed across intensive (57.9%), extensive (26.3%) and organic soils (15.8%) (Figure 7.1). *E. coli* represented 31.6% of this isolates, followed by 26.3% of *Pseudomonas* spp., 15.8% of *Stenotrophomonas* spp., 7.9% for each of the *Achromobacter* spp. and *Aeromonas* spp. genera, 5.3% of *Acinetobacter* and 2.6% for each of *Alcaligenes* spp. and *Citrobacter* spp. genera. Additionally, 13.8% of the isolates were presumptively producers of metallo-β-lactamases: 62.5% correspond to *Pseudomonas* spp. and 37.5% to *Stenotrophomonas* spp.; among these 28.1% were also phenotypically positive for the production of ESBL (Figure 7.1).

Correlation of antibiotic nonsusceptibility with agricultural practices

Odds ratios were used to compare the relative odds of the occurrence of antibiotic nonsusceptible isolates, given exposure to different agricultural practices and seasons.

This analysis identified variables significantly and independently associated with antibiotic nonsusceptibility, presence of multidrug resistance or production of ESBL (Table 7.2).

Although a positive correlation has been detected between organic agricultural settings with both chloramphenicol ($p \leq 0.01$) and nitrofurantoin ($p \leq 0.01$), this type of setting was mainly negatively associated with antibiotic nonsusceptibility (Table 7.2). Indeed, a protective association was found between organic agricultural practice and nonsusceptibility to several antibiotics, regardless of the season: streptomycin ($p = 0.05$), norfloxacin ($p \leq 0.01$), ciprofloxacin ($p \leq 0.01$), amikacin ($p \leq 0.01$) and tetracycline ($p \leq 0.01$). Organic soils also provided a protection factor for the production of ESBL ($p \leq 0.01$). In contrast, intensive soil samples represented a risk factor for nonsusceptibility to a greater number of antibiotics. These soils were significantly and positively correlated with nonsusceptibility to cefepime ($p \leq 0.01$), norfloxacin ($p \leq 0.01$), ciprofloxacin ($p = 0.02$), gentamicin ($p \leq 0.01$), streptomycin ($p \leq 0.01$) and tetracycline ($p \leq 0.01$), and with production of ESBL ($p \leq 0.01$).

The extensive agricultural practice constituted a risk factor for the existence of isolates nonsusceptible to fluoroquinolones (norfloxacin and ciprofloxacin, $p = 0.02$ and $p \leq 0.01$) in the winter season; in contrast, these soils were negatively associated with carbapenems ($p \leq 0.01$), nalidixic acid ($p \leq 0.01$), and tetracycline ($p = 0.02$). Overall, the summer season constituted a risk factor for the occurrence of isolates nonsusceptible to kanamycin ($p \leq 0.01$), chloramphenicol ($p \leq 0.01$) and nitrofurantoin ($p \leq 0.01$); the winter season constituted a risk factor for the appearance of ciprofloxacin nonsusceptibility ($p \leq 0.01$).

Analyses of the intrinsic resistance

We identified 134 clinically important isolates for which we evaluated and analyzed the intrinsic nonsusceptibility (Table 7.3). The profiles of intrinsic resistance of soil bacteria were quite different (either using species specific or nonspecific breakpoints) when compared to what is described (Bonnet et al., 2013): apart from *Citrobacter freundii* and *Providencia retgerri*, all clinically important isolates recovered from soil revealed intrinsic resistance to more antibiotics than what is described for its clinical counterparts (Table 7.3). In fact, all the nonfermenting Gram negative bacilli (*Pseudomonas aeruginosa*, *Acinetobacter* spp., *Stenotrophomonas malthophilia*, *Achromobacter* spp. and *Ochrobactrum anthropi*) showed additional intrinsic resistance to up to 6 different groups of antibiotics, being cephamycins present in all of them (Table 7.3). Moreover, *P. aeruginosa* isolates recovered from soil did not present intrinsic resistant to carbapenems.

Analyses of antibiotic resistance genes-producing isolates

We have detected 12 *E. coli*, out of 232 isolates (5.2%), harbouring mobile genes encoding β-lactamases from four different families (TEM, SHV, CTX-M and CMY), including $bla_{\text{TEM-135}}$ (n=1), $bla_{\text{TEM-1}}$ (n=9), $bla_{\text{SHV-12}}$ (n=2), $bla_{\text{CMY-2}}$ (n=1) and $bla_{\text{CTX-M-1}}$ (n=7) (Table 7.4). In one isolate, $bla_{\text{TEM-type}}$ gene was co-produced with one $bla_{\text{SHV-12}}$, in other with $bla_{\text{CMY-2}}$, and in six other isolates with $bla_{\text{CTX-M-1}}$. The *bla*-harbouring isolates also expressed nonsusceptibility to two to five different classes of antibiotics (Table 7.4), and were isolated from the winter sampling of sample SL2, which comprises a large intensive production. All the remaining isolates (n=220) were negative to these and other *bla* genes investigated, including the ones that showed phenotypes indicative of β-lactamase production.

Epidemiology and diversity of *E. coli* isolates

The 12 *E. coli* isolates belonged to nine different STs, from which ST58 and ST155 belong to clonal complex (CC) CC155, and ST90 to CC23; six other singletons were also identified. Isolate EcAmb111 was assigned a new allele combination, which was registered in the MLST database as ST4493.

Characterization of *bla* associated mobile genetic elements

The PBRT typing method showed a predominance of IncI1 plasmids (n=11) among *bla* genes-harbouring isolates, followed by FIB (n=9) and FrepB (n=2) (Table 7.4). Conjugation results revealed that all isolates were only able to transfer IncI1 plasmids (n=11), showing

that the all *bla* genes transferred were contained there, with exception of bla_{TEM-1} of isolate EcAmb160 that was harboured by a FIB plasmid (Table 7.4). Actually, the only bla_{TEM} genes transferred belonged to isolates EcAmb160 and EcAmb268, which did not harbor any other *bla* resistance genes. The two bla_{SHV-12}-carrying IncI1 plasmids were assigned to ST29 and the bla_{CMY-2}-carrying plasmid was assigned to ST2. Five out of seven $bla_{CTX-M-1}$-carrying plasmids were assigned to ST3, while the remaining two were assigned to ST28, all belonging to clonal complex 3. The only positively transferred bla_{TEM-1}-carrying IncI1 plasmid was assigned to ST21. The determination of the upstream genetic context of *bla* genes showed the presence of IS*Ecp1* of all $bla_{CTX-M-1}$ (n=7) and bla_{CMY-2} (n=1), being bla_{SHV-12} (n=2) flanked by IS*26*. The downstream genetic context of the genes consisted of *orf477* in all $bla_{CTX-M-1}$ genes (n=7), IS*26* in bla_{SHV-12} (n=2) and a *blc-sugE* structure in bla_{CMY-2} (n=1) (Table 7.4). Five out of the eleven bla_{TEM} genes were associated to *tn3*, including the $bla_{TEM-135}$ gene, while other three were directly associated to IS*26*. The remaining two bla_{TEM-1} genes were not associated to any of the investigated elements.

7.5. Discussion

The current study demonstrated the widespread presence of high-level antibiotic-resistant bacteria (nearly 86% of MDR) in all sampled soils, suggesting that the type of agriculture practice might not directly influence or enhance the global levels of resistance. However, striking differences were noticed in intensive soils for norfloxacin (74%), streptomycin (50.7%) and tetracycline (46.6%).

While protective associations were mainly guaranteed by organic agriculture, positive correlations were mostly noticed in the analysis of the intensive soils. Indeed, the exposure to the intensive agricultural practice constituted a risk factor for nonsusceptibility to many antibiotics, multidrug resistance and production of ESBL (Table 7.2). Although there is no record of antibiotic use for the sampled soils, the intensive agriculture locations have been long used for intensive farming with resource to amendments and pesticides, which might have contributed to this discrepancy. Indeed, Popowska et al. (2012) have also detected high levels of tetracycline and streptomycin resistance, as well as elevated rates of MDR in bacteria isolated from manure-amended soils or soils from agricultural systems (Popowska et al., 2012).

We have also detected nonsusceptibility for last resource antibiotics such as carbapenems (Figure 7.2). In fact, considering that intrinsic nonsusceptibility to those antibiotics was only detected in two groups of clinically important bacteria (*Acinetobacter* spp. and

Stenotrophomonas spp.), it suggests that many other environmental bacteria do harbor this resistance, for instance due to the production of chromosome-encoded carbapenemases, making it available to recruitment by pathogenic bacteria that co-inhabits the soil ecosystem (Rossolini et al., 2001; Saavedra et al., 2003). Moreover, the production of rare non mobile β-lactamases might justify the rate of phenotypic ESBL/MBL detection in this study.

The low levels of resistance to fluoroquinolones for isolates from organic soil settings are also worth mentioning; in fact, it is not likely that our organic locations have been exposed to these synthetic antibiotics, since not long ago they were considered a pristine environment, and are still located within a natural park. Thus, there was no selection pressure for the development of fluoroquinolone resistance, as opposed to extensive and intensive sites, which might have previously been exposed through wastewater or manure from animal husbandries.

Moreover, our study described unusual low levels of nonsusceptibility to gentamicin and amikacin, when compared with other studies (Walsh and Duffy, 2013); indeed, this suggests that intrinsic and acquired aminoglicosydes resistance mechanisms might be more widespread among Gram positive and non culturable bacteria, than in Gram negative bacteria (Riesenfeld et al., 2004; Bonnet et al., 2013).

Globally, the determination of the intrinsic resistance profiles from the isolates recovered from soil (Table 7.4) revealed that they were resistant to more antibiotic classes than what is described for that species or genera (Bonnet et al., 2013), which might suggest that intrinsic resistance might not be coherent between bacteria acquired in healthcare settings and bacteria acquired from other settings, particularly environment. This fact might raise an important concern, because definition of therapy takes into account the described intrinsic resistance patterns, which might thus be different in infectious acquired from non clinical sources (Kollef, 2005; Cox and Wright, 2013). We should also consider the relevance of environment-borne infections in clinical settings, and eventually define species specific breakpoints for opportunistic environmental bacteria, since they might represent a health threat.

Today, we know that approximately only 1% of bacteria can be cultured under laboratory conditions (Walsh and Duffy, 2013). In fact, despite the fact that culture techniques may always bias the population of bacteria recovered, they provide the advantage of being able to evaluate the phenotypes, and study the biological context of the mechanisms of resistance with regards to genetic support and hosts, which can be helpful to track them.

Table 7.3. Comparative intrinsic nonsusceptibility[a] profiles of different clinically important Gram negative bacteria (n=134) recovered from soil samples.

Bacteria	Number of isolates	Intrinsic nonsusceptibility of soil isolates (n=134) according to:		Intrinsic nonsusceptibily profile according to SFM[c]
		Species nonspecific breakpoints[c]	Species specific breakpoints[c]	
Enterobacter spp.	9	PEN, I, 1GC, 2GC, F	PEN, I, 1GC, 2GC, F	PEN, I, 1GC, F
Citrobacter freundii	14	PEN	NI	PEN, I, 1GC, F
Escherichia coli	23	I, 2GC	NI	NA
Providencia retgerri	3	FT	FT	PEN, I, 1GC, F, TE, FT
Proteus mirabilis	3	TE,Q, FT	TE,Q, FT	TE, FT
Proteus vulgaris	1	PEN, 1GC, 2GC , Q, SXT, FT, TE	PEN, 1GC, 2GC , Q, SXT, FT, TE	PEN, 1GC, 2GC, FT, TE
Pseudomonas aeruginosa	2	PEN, 1GC, 2GC, 3GC, F, Q, A, TE, PL, FT, SXT	NI	PEN, 1GC, 2GC, 3GC, C, Q, A, TE, PL, SXT
Acinetobacter baumannii/calcoaceticus[b]	28	PEN, 1GC, 2GC, 3GC, I, M, F, C, Q, PL, FT	3GC, PL	PEN, 1GC, 2GC, M, C, FT, SXT
Stenotrophomonas malthophilia[b]	14	PEN, 1GC, 2GC, 3GC, M, C, F, Q, FT	C	PEN, 3GC, C
Achromobacter spp.[b]	16	2GC, 3GC, M, F, Q, FT	NI	3GC
Ochrobactrum anthropi[b]	13	PEN, 1GC, 2GC, 3GC, 4GC, I, M, F, Q, A, FT, PL	NI	PEN, 1GC, 2GC, 3GC, 4GC, I
Aeromonas spp.[b]	8	PEN, 1GC, 2GC, I	NI	PEN, 1GC, 2GC, C

[a] Intrinsic nonsusceptibility was defined as nonsusceptibility of greater or equal of 80% of the studied population, except for *P. vulgaris* and *P. aeruginosa* due to the reduced number of isolates.

[b] Species specific breakpoints were not available for many or all of the antibiotics tested for these species.

[c] According to Bonnet et al. 2013; differences between soil bacteria and described intrinsic nonsusceptibility profile are underlined.

NI, No intrinsic nonsusceptibilities were detected using the available species specific breakpoints for the tested antibiotics.

NA, No intrinsic nonsusceptibility profile described for this species (Bonnet et al. 2013).

PEN, penicillins (amoxicillin, ticarcillin); 1GC, first generation cephalosporins (cephalothin); 2GC, second generation cephalosporins (cefuroxime); 3GC, third generation cephalosporins (cefotaxime, ceftazidime, ceftriaxone, cefpodoxime); 4GC, fourth generation cephalosporins (cefepime); M, monobactam (aztreonam); C, carbapenems (imipenem, meropenem, ertapenem); I, β-lactam with β-lactamase inhibitor combinations (amoxicillin plus clavulanic acid, piperacillin plus tazobactam); F, cephamycins (cefoxitin); PL, phenicols (chloramphenicol); Q, quinolones (nalidixic acid, norfloxacin, ciprofloxacin); A, amynoglicosides (gentamicin, kanamycin, amikacin, streptomycin); SXT, sulphonamides (trimethoprim plus sulfamethoxazole); TE, tetracyclines (tetracycline); FT, nitrofurans (nitrofurantoin).

Table 7.4. Phenotypic and genotypic profile of *bla* mobile genes and plasmids[a] detected in 12 *E. coli* isolates from winter sampling of soil SL2.

Isolate	Antibiotic susceptibility														*bla* genes	Genetic context	Plasmids	pMLST IncI1 (CC)	MLST (CC)
	PEN	1GC	2GC	3GC	4GC	M	C	I	F	PL	Q	A	SXT	TE	FT					
EcAmb106																*bla*$_{TEM-1}$+ *bla*$_{CTX-M-1}$	IS*26*-*bla*$_{TEM-1}$ ISE*cp1*-*bla*$_{CTX-M-1}$-*orf477*	IncI1, FIB	ST3 (CC3)	ST2025
EcAmb111																*bla*$_{TEM-1}$+ *bla*$_{SHV-12}$	IS*26*-*bla*$_{SHV-12}$-*IS26*	IncI1, FrepB	ST29 (CC26)	ST4493
EcAmb112																*bla*$_{SHV-12}$	IS*26*-*bla*$_{SHV-12}$-*IS26*	IncI1, FrepB	ST29 (CC26)	ST155 (CC155)
EcAmb115																*bla*$_{TEM-1}$+*bla*$_{CMY-2}$	*tn3*-*bla*$_{TEM-1}$ IS*Ecp1*-*bla*$_{CMY-2}$-*blc*-*sugE*	IncI1, FIB	ST2 (CC2)	ST683
EcAmb157																*bla*$_{TEM-1}$+ *bla*$_{CTX-M-1}$	ISE*cp1*-*bla*$_{CTX-M-1}$-*orf477*	IncI1, FIB	ST3 (CC3)	ST2025
EcAmb160																*bla*$_{TEM-1}$	IS*26*-*bla*$_{TEM-1}$	FIB	ND	ST58 (CC155)
EcAmb268																*bla*$_{TEM-1}$	IS*26*-*bla*$_{TEM-1}$	IncI1, FIB	ST21 (CC5)	ST90 (CC23)
EcAmb269																*bla*$_{CTX-M-1}$	ISE*cp1*-*bla*$_{CTX-M-1}$-*orf477*	IncI1	ST3 (CC3)	ST2687
EcAmb271																*bla*$_{TEM-1}$+ *bla*$_{CTX-M-1}$	*tn3*-*bla*$_{TEM-1}$ ISE*cp1*-*bla*$_{CTX-M-1}$-*orf477*	IncI1, FIB	ST3 (CC3)	ST58 (CC155)
EcAmb272																*bla*$_{TEM-1}$+ *bla*$_{CTX-M-1}$	*tn3*-*bla*$_{TEM-1}$ ISE*cp1*-*bla*$_{CTX-M-1}$-*orf477*	IncI1, FIB	ST3 (CC3)	ST906
EcAmb275																*bla*$_{TEM-1}$+ *bla*$_{CTX-M-1}$	*tn3*-*bla*$_{TEM-1}$ ISE*cp1*-*bla*$_{CTX-M-1}$-*orf477*	IncI1, FIB	ST28 (CC3)	ST58 (CC155)
EcAmb278																*bla*$_{TEM-135}$+ *bla*$_{CTX-M-1}$	*tn3*-*bla*$_{TEM-135}$ ISE*cp1*-*bla*$_{CTX-M-1}$-*orf477*	IncI1, FIB	ST28 (CC3)	ST1718

Black, resistance; dark grey, intermediate resistance; light grey, susceptibility.

[a] Genes and plasmids present in transformants are underlined.

PEN, penicillins (amoxicillin, ticarcillin); 1GC, first generation cephalosporins (cephalothin); 2GC, second generation cephalosporins (cefuroxime); 3GC, third generation cephalosporins (cefotaxime, ceftazidime, ceftriaxone, cefpodoxime); 4GC, fourth generation cephalosporins (cefepime); M, monobactam (aztreonam); C, carbapenems (imipenem, meropenem, ertapenem); I, β-lactam with β-lactamase inhibitor combinations (amoxicillin with clavulanic acid, piperacillin with tazobactam); F, cephamycins (cefoxitin); PL, phenicols (chloramphenicol); Q, quinolones (nalidixic acid, norfloxacin, ciprofloxacin); A, amynoglicosides (gentamicin, kanamycin, amikacin, streptomycin); SXT, sulphonamides (trimethoprim with sulfamethoxazole); TE, tetracyclines (tetracycline); FT, nitrofurans (nitrofurantoin).

In fact, despite the fact that culture techniques may always bias the population of bacteria recovered, they provide the advantage of being able to evaluate the phenotypes, and study the biological context of the mechanisms of resistance with regards to genetic support and hosts, which can be helpful to track them.

Five different *bla* genes from three different families were detected among 12 *E. coli* isolates recovered from winter sampling of soil SL2: $bla_{\text{TEM-1}}$, $bla_{\text{TEM-135}}$, $bla_{\text{SHV-12}}$, $bla_{\text{CMY-2}}$ and $bla_{\text{CTX-M-1}}$ (Table 7.4). Although $bla_{\text{TEM-1}}$ is widely reported in clinical, veterinary and environmental settings (Leverstein-van Hall et al., 2011; Bardoň et al., 2013), to the best of our knowledge this constitutes the first description of $bla_{\text{SHV-12}}$ and $bla_{\text{TEM-135}}$ and one of the first reports of $bla_{\text{CTX-M}}$ and bla_{CMY} gene in soils.

The epidemiologic characterization of ESBL-producing *E. coli* showed that, despite the previous appearance of clonally related $bla_{\text{CTX-M}}$-harbouring isolates in soils (Hartmann et al., 2012), the isolates in this study were genetically diverse. We have detected nine different STs distributed among two clonal complexes (ST23 and ST155) and six singletons, including the novel ST4493, which suggests that dissemination of resistance determinants was probably mainly carried out by horizontal gene transfer. In fact, the mobilization of the detected *bla* genes was driven by five different IncI1 plasmids: ST2 associated to $bla_{\text{CMY-2}}$, ST3 and ST28 associated to $bla_{\text{CTX-M-1}}$, ST21 associated to $bla_{\text{TEM-1}}$ and ST29 associated to $bla_{\text{SHV-12}}$. Indeed, $bla_{\text{CTX-M-1}}$ was associated to two different plasmids from clonal complex 3, being uncertain if it represents intra-plasmid evolution or plasmid acquisition from multiple sources. ST2/IncI1 and ST3/IncI1 have been extensively associated to the spread of CTX-M-1 and CMY-2 β-lactamases in food-producing animals, which represents a concern, because manure amendments might disseminate it through agriculture soils, and vegetable products (Grami et al., 2013; Rodrigues et al., 2013; Haenni et al., 2014).

The characterization of the flaking regions of the *bla* genes reinforced the results of plasmid characterization, revealing the same genetic background and the same mobile genetic elements (IS*Ecp1*, IS*26* and tn*3*) for soil isolates than what is described for animal and human isolates (Jones-Dias et al., 2013; Wang et al., 2014). To date, very few studies have performed an in-depth analysis of mobile elements associated with clinically important *bla* genes in soil ecosystems. Ultimately, both our study and Ben Said et al. (2015) reflect that the *bla*-harboring isolates detected share their molecular background with isolates from human and/or animal sources, pointing to contamination of agricultural soils.

Although the nonsusceptibility levels varied between different agricultural practices, soils, species and antibiotics, the presence of IncI1-driven spread of penicillinases ($bla_{\text{TEM-1}}$,

bla$_{\text{TEM-135}}$), ESBL (*bla*$_{\text{SHV-12}}$ and *bla*$_{\text{CTX-M-1}}$) and PMAβ (*bla*$_{\text{CMY-2}}$) were only associated with intensive agriculture, particularly in the winter season of soil SL2, which comprises a large scale monoculture production of tomatoes, a vegetable that is among one of the most consumed fresh produces in Portugal. Thus, our data suggests that pathogenic bacteria might be introduced in soil through manure amendments or other contaminated sources related to intensive farming. The soil ecological dynamic may be threatened by the introduction of pathogenic microorganisms, which may provide harmless soil-dwelling bacteria, not only with additional antibiotic resistance genes, but also with determinants associated with the establishment of pathogenicity and the transfer of foreign DNA.

It would be important to evaluate the extent of this scenario by analyzing the Gram negative population contained in fresh vegetables from different types of agricultural practices.

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Chapter 8.

Architecture of class 1, 2 and 3 integrons from Gram negative bacteria recovered among organic and conventionally produced fruits and vegetables

This research paper was submitted as:

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Contributions of the authors for the following manuscript:

Daniela Jones-Dias: conception and design of study, acquisition of laboratory and epidemiological data, analysis of data, drafting of article and critical revision of the manuscript, final approval of manuscript;*
Vera Manageiro: analysis of data, drafting of article and critical revision of the manuscript, final approval of manuscript;*
Eugénia Ferreira: acquisition of laboratory data, final approval of manuscript;
Paula Barreiro: acquisition of laboratory data, final approval of manuscript;
Luís Vieira: acquisition of laboratory data, final approval of manuscript;
Inês Barata Moura: acquisition of laboratory data, final approval of manuscript;
Manuela Caniça: conception and design of study, critical revision of the manuscript, final approval of manuscript.

** these authors contributed equally to this work.*

8.1. Abstract

The spread of antibiotic resistant bacteria throughout the food chain constitutes a public health concern. In this study, we showed that organic and conventionally produced fruits and vegetables contained antibiotic resistant Gram negative isolates (n=333), of which many were bacteria that can act as human commensals and opportunistic pathogens. However, analyses of antibiotic nonsusceptibility showed no statistically significant correlation between antibiotic nonsusceptibility and mode of foodstuff production. The genomic characterization of integron-harboring isolates revealed an impressive number of mobile genetic elements and clinically relevant acquired antibiotic resistance genes, such as *mcr-1*, *qnrA1*, $bla_{\text{GES-11}}$, *mphA* and *oqxAB*. The study of class 1 (n=8), class 2 (n=3) and class 3 (n=1) integrons, harboured by species such as *Morganella morganii*, *Escherichia coli* or *Klebsiella pneumoniae* led to the identification of different promoters (PcW, PcH1, PcS and $\text{PcW}_{\text{TNG-10}}$) and cassette arrays (containing *drfA*, *aadA*, *cmlA, estX, sat,* bla_{GES}). In fact, the diverse integron backbones were associated with transposable elements (e.g. Tn*402*, Tn*7*, *ISCR*1, Tn*2**, IS*26*, IS*1326* and IS*3*) that conferred greater mobility. This is also the first appearance of In*1258*, In*1259* and In*3-13*, which should be monitored to prevent their establishment as successfully dispersed mobile resistance integrons. These results underscore the growing concern about the dissemination of acquired resistance genes by mobile elements in the food chain.

8.2. Introduction

The spread of antimicrobial resistance has made bacterial infections gradually more difficult to treat (Blair et al., 2015; Hawkey, 2015). Increasing evidence suggests that intestinal microbiota of humans and animals constitute a reservoir for antibiotic resistant isolates and resistance genes (Hu et al., 2014). In fact, antibiotic resistant community- and hospital-acquired infections are often caused by bacteria that may inhabit the human gut (Cantas et al., 2013). Consequently, a better understanding on how antibiotic resistant isolates break into the human microbiota is essential to prevent multidrug resistant infections.

Fresh produce frequently harbor nonpathogenic environmental microorganisms (Aserse et al., 2013). During growth and harvesting, vegetables and fruits can also become contaminated with pathogenic and commensal bacteria from animals and humans. This contamination can occur in the field through direct contact with soil, and through the application of manure and wastewater as biofertilizers (Ben Said et al., 2015; Berendonk

et al., 2015; van Hoek et al., 2015). In addition, crops are subjected to high selection pressure caused by exposure to antibiotic residues indirectly coming from manure and wastewater, or directly from phytopharmaceutical agents (Gaze et al., 2011; Finley et al., 2013). Thus, fruits and vegetables that are grown close to the soil, and that are not subjected to any type of cooking process are especially prone to transmit contaminant microorganisms (Berger et al., 2010).

In Portugal, the majority of the fruits and vegetables that are available to the consumers are conventionally produced (GPP, 2011). However, organic produce constitute a viable alternative that is getting more supporters every year in western countries (GPP, 2011; Jensen et al., 2011). According with the European rules, although organic production does not comprise the use of chemical agents, it still allows the use of manure from farming and sewage (EC, 1991). The overall influence of conventional and organic produce in the exposure of consumers to resistant bacteria has been evaluated in France in 2009, showing that those contained equivalent amounts of antibiotic resistant Gram negative bacteria (Ruimy et al., 2010). However, a recent study from Portugal that analyzed different agricultural soil samples, revealed that exposure to conventional agricultural practices constituted a risk factor for nonsusceptibility to many antibiotics, multidrug resistance and production of Extended-Spectrum β-Lactamases (ESBL) (Jones-Dias et al., 2016).

Horizontal transfer of genetic material among Gram negative bacteria plays an important role in the dissemination of multidrug resistance. The location of antibiotic resistant genes on mobile genetic elements, such as plasmids, transposons, and integrons, facilitates the mobilization of resistance among microorganisms (Stokes and Gillings, 2011). The latter elements are mainly comprised of an integrase gene (*intI*) whose product allows them to capture and collect gene cassettes through a recombination site (*attI*). In turn, the integron itself may be directly associated with transposons, which enhances their mobility in the cell, such as transposons 402 (Tn*402*) in class 1 integrons and transposons 7 (Tn*7*) in class 2 integrons (Cambray et al., 2010; Stokes and Gillings, 2011). Classes 1, 2 and 3 integrons display elevated clinical importance, being responsible for the ongoing accumulation of genes cassettes coding for antibiotic resistance genes (Escudero et al., 2015). Recently, a study by Gillings et al. (2015) proposed the use of class 1 integrase-encoding gene as a generic marker for anthropogenic pollutants, due to its common association with antibiotic resistance and heavy metals, and its appearance in pathogenic and non-pathogenic bacteria (Gillings et al., 2015).

Therefore, the main objectives of this study were to assess the information on antibiotic resistance bacteria gathered from organically and conventionally grown fruits and

vegetables produced and marketed in Portugal, and to characterize the diversity of class 1, 2 and 3 integrons detected within these fresh produce, relating their architecture with accumulation and spread of antibiotic resistance throughout the food chain.

8.3. Materials and methods

Fresh produce sampling

Between March 2013 and February 2014, one conventional and one organic batch of three fruits and three vegetables, produced and marketed in Portugal, was purchased at retail stores in the region Lisbon and Tagus Valley, once a month, as available (Table 8.1). The specimens were meant to represent the most consumed fresh produce in Portugal. Organic products were either bought at certified specialized stores, or were differentiated from conventional produce by the presence of specific labels, indicating that the producer respects specific regulation. Overall, we evaluated 144 products, including 74 vegetables and 70 fruits. Of these, 24 were green leaf lettuces (*Lactuca sativa* var. *romana*), 26 tomatoes (*Solanum lycopersicum*), 24 carrots (*Daucus carota*), 20 pears (*Pyrus communis*), 26 apples (*Malus* spp.) and 24 were strawberries (*Fragaria* spp). The exact same number of samples was collected from conventional and organic products (72/144, 50%). All products were immediately transported to the laboratory and processed: 50g of each product was selected at random without washing or peeling, diluted 1:5, homogenized (Stomacher 80 Biomaster®, Seward, UK), labeled, and frozen at -80°C.

Selection and identification of antibiotic resistance bacteria

The selection of 333 resistant Gram negative bacteria was performed in violet red bile glucose agar (VRBG) plates containing specific concentrations of different antibiotics: 100mg/L of amoxicillin, 2mg/L of cefotaxime, 2mg/L of ceftazime, 4mg/L of ertapenem, 2mg/L of imipenem, 10mg/L of tetracycline, 20mg/L of chloramphenicol, 50mg/L of nalidixic acid, 4mg/L of ciprofloxacin or 2mg/L of gentamicin. Individual colonies were then selected based on their different morphology. The isolates were identified through the amplification of the 16*S* ribosomal RNA (rRNA) genes using bacterial 16*S* primers: 8F 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R 5'-GGTTACCTTGTTACGACTT-3'; for isolates that no PCR product was produced a second set of primers was used 16SF 5'-AGAGTTTGATCMTGGCTCAG-3' and 16SR 5'-GTAAGGTTCTKCGCGTTGC-3'. PCR products were then purified with ExoSAP IT (USB Corporation, Cleveland, OH), and further sequenced directly, on both strands, using automatic sequencer ABI3100 (Applied

Biosystems, Warrington, UK). The resulting sequences were then analyzed using the Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium) and assigned to respective identification, using the tools available on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Antibiotic susceptibility testing

Antibiotic susceptibility was analyzed for 308 isolates, distributed among *Enterobactericeae*, *Acinetobacter* spp. (family *Moraxellaceae*) and *Pseudomonas* spp. (family *Pseudomonadaceae*). This analysis was performed by disc diffusion method against the following antibiotics (Biorad): amoxicillin (25 µg), ampicillin (10 µg), cefotaxime (5 µg), ceftazidime (10 µg), cefepime (30 µg), cefoxitin (30 µg), ertapenem (10 µg), imipenem (10 µg), amoxicillin with clavulanic acid (20+10 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), gentamicin (15 µg), amikacin (30 µg), chloramphenicol (75 µg), tetracycline (30 µg), and trimethoprim with sulfamethoxazole (1.25+23.75 µg). Interpretation of results was performed according to the cut-off values recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, http://mic.eucast.org/Eucast2/). The breakpoints of the Antibiogram Committee of the French Society of Microbiology (CA-SFM, http://www.sfm-microbiologie.org/) were applied, when available, for antibiotics that were not predicted by EUCAST. These guidelines showed no available breakpoints for microorganisms belonging to *Delftia* spp., *Aeromonas* spp. and *Comamonas* spp., and for this reason, they (n=12) were not included in the assessment of antibiotic nonsusceptibility. Susceptibility of isolates from genera *Stenotrophomonas* spp. (n=13) was interpreted through the only EUCAST or CA-SFM available.

Double disk synergy test was used to phenotypically detected the presence of specific groups of β-lactamases in the 333 isolates: amoxicillin (25 µg) and amoxicillin with clavulanic acid (20+10 µg) for the phenotypic detection of penicillinases, cefotaxime (5 µg) and amoxicillin with clavulanic acid (20+10 µg) for extended-spectrum β-lactamases (ESBL), cefoxitin (30 µg) and cloxacillin (750 µg) for AmpC β-lactamases, and imipenem (5 µg) and dipicolinic acid (750 µg) for metallo-β-lactamases (MBL). *E. coli* ATCC® 25922™ was also tested as a control of this technique.

Statistical analysis of nonsusceptibility results

We tested for positive and negative associations between each type of agricultural practice (conventional and organic) and nonsusceptibility to different antibiotics, multidrug resistance, and phenotypic production of β-lactamases. The following antibiotics were

tested: ceftazidime, cefepime, imipenem, ciprofloxacin and gentamicin (Table S8.1). OpenEpi software, version 3.03a was used for statistical analysis (Dean et al., 2015). Fisher exact test was used to assess differences in antibiotic nonsusceptibility between different groups. Associations were determined by calculation of odds ratios with 95% confidence intervals. The null hypothesis was rejected for *p* values of ≤0.05.

Molecular detection of class 1, 2 and 3 integrons

All isolates were investigated for the presence of class 1, 2 and 3 integrase-encoding genes, through PCR amplification using primers reported elsewhere (Leverstein-Van Hall et al., 2002; Corrêa et al., 2014; Manageiro et al., 2015a).

Whole genome sequencing of integron-carrying isolates

All integrase-positive isolates were characterized by whole genome sequencing. Briefly, genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Aarhus) and quantified using Qubit 1.0 Fluorometer (Invitrogen, Waltham). The Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA) was used to prepare sequencing libraries from 1ng of genomic DNA according to the manufacturer's instructions. Paired-end sequencing of 250 bp reads was performed on a MiSeq (Illumina). Analysis of the integrase-producing isolates was carried out as described elsewhere (Jones-Dias et al., 2015a). The NCBI prokaryotic genome automatic annotation pipeline (PGAAP) was used for annotation (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/). Specific analysis of class 1, 2 and 3 integrons was carried out with CLC genomics workbench version 8.5.1 (Qiagen). Contigs carrying integrons were manually assembled and annotated whenever was necessary. Isolates showing novel MLST alleles combinations were assigned to sequence types (STs) through the respective MLST websites (http://pubmlst.org/ecloacae/ and https://enterobase.warwick.ac.uk/).

Nucleotide sequence accession numbers

This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession PRJNA311932. The versions described in this paper are the following: LSUR00000000 (*Enterobacter cloacae* INSali2), LSUS00000000 (*E. cloacae* INSali10), LSRK00000000 (*E. coli* INSali25), LSUT00000000 (*E. coli* INSali38), LSUU00000000 (*E. coli* INSali92), LSUV00000000 (*Raoultella ornithinolytica* INSali127), LSUW00000000 (*R. ornithinolytica* INSali133), LSUX00000000 (*M. morganii* INSali207), LSUY00000000 (*E.*

coli INSali370), LSUZ00000000 (*Pseudomonas putida* INSali382) and LSVA00000000 (*K. pneumoniae* INSali390).

Integrons sequence submission

The integron sequences were submitted to the INTEGRALL database (http://integrall.bio.ua.pt) for integron number assignment.

8.4. Results

Gram negative population of fresh fruits and vegetables

Three hundred and thirty three Gram negative antibiotic resistant isolates, cultured from 72 conventionally and 72 organically produced fruits (n=70) and vegetables (n=74) were obtained upon selection with the antibiotics previously referred (Table 8.1).

Table 8.1. Distribution of 144 samples (70 fruits and 74 vegetables) according with date of collection, store and produce.

Date of collection (Month/ Year)	Conventional		Organic	
	Store code	Produce	Store code	Produce
March, 2013	F	Ap, Cr, Le, Pe, St, To	C	Ap, Cr, Le, Pe, To
April, 2013	I	Ap, Cr, Le, Pe, St, To	E	Ap, Cr, Le, To, St
May, 2013	G	Ap, Cr, Le, St, To	C	Ap, Cr, Le, St, To
June, 2013	L	Ap, Cr, Le, St, To	K	Ap, Cr, Le, St, To
July, 2013	C	Ap, Pe, St, To	G	Cr, Le, Pe, To
August, 2013	A	Ap, Le, St, To	J	Ap, Cr, Le, St, Pe
September, 2013	L	Ap, Cr, Le, Pe, To	B	Ap, Le, Pe, To
October, 2013	H	Ap, Le, Pe, To	D	Ap, Cr, Le, Pe, To
November, 2013	G	Ap, Cr, Le, Pe, To, St	G	Ap, To
December , 2013	J	Ap, Le, Pe, To	A	Ap, Le, Pe, To
January, 2014	J	Cr, Pe, St	E	Cr, Le
February , 2014	J	Ap, Cr, St, To	C	Cr, Le, St

Ap, Apple (n=26); Cr, Carrot (n=20); Le, Lettuce (n=24); Pe, Pear (n=20); St, Strawberry (n=24); To, Tomato (n=26).

16S rDNA profiling revealed that the isolates were represented by 20 bacterial genera, distributed among six main families of Gram negative bacteria, among which the predominant was *Enterobactericeae* (55.3%) [*Enterobacter* (23.4%), *Serratia* (7.2%), *Raoultella* (5.4%), *Klebsiella* (4.5%), *Rahnella* (4.5%), *Escherichia* (3.0%), *Morganella* (2.4%), *Citrobacter* (1.5%), *Providencia* (1.5%), *Pantoeae* (0.6%), *Cedecea* (0.3%), *Proteus* (0.3%), *Leclercia* (0.3%) and *Erwinia* (0.3%)] (Figure 8.1). The remaining groups

comprised *Moraxellaceae* (*Acinetobacter*, 26.7%), *Pseudomonadaceae* (*Pseudomonas*, 10.5%), *Xanthomonadaceae* (*Stenotrophomonas*, 3.9%), *Comamonadaceae* (1.8%) [*Delftia* (0.9%) and *Comamonas* (0.9%)], and *Aeromonadaceae* (*Aeromonas*, 1.8%) (Figure 8.1). The bacterial genera detected in the assessed fresh produce could be traced back to different origins: although some genera such as *Delftia* spp., *Erwinia* spp. and *Comamonas* spp. are mainly environmental, the majority of the genera comprised bacteria that are often described as commensal and clinically relevant, such as *E. coli* and *K. pneumoniae* (Figure 8.1). Overall, most of the identified taxa (e.g. *Leclercia* spp., *Pantoeae* spp.) included bacterial genera that are usually recovered from the environment but are also able to cause infections (Guentzel, 1996; Marshall et al., 2009).

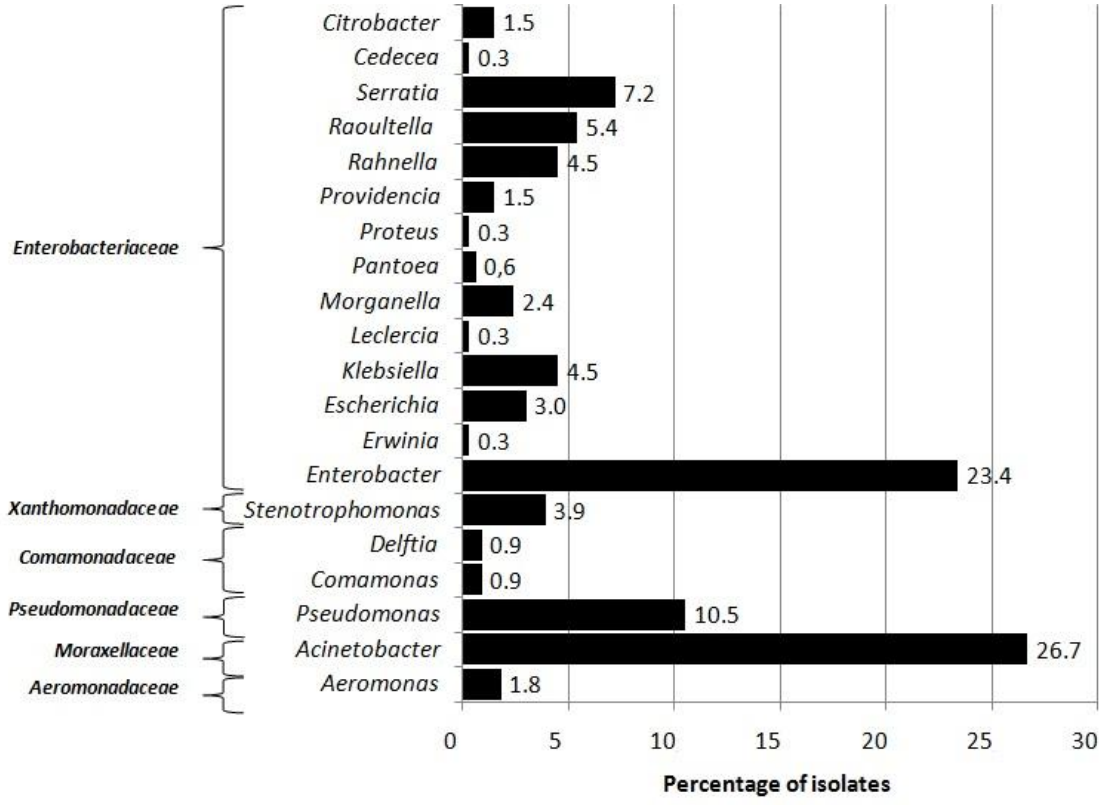


Figure 8.1. Percentage of bacterial genera recovered from fruits and vegetables.

Evaluation of antibiotic susceptibility

For *Enterobactericeae* (n=184), β-lactam nonsusceptibility ranged between 0% for imipenem/cefepime and 45.1% for ampicillin, in isolates from conventionally produced fruits and vegetables (Table 8.2). Small variations regarding β-lactam nonsusceptibility

were detected among the modes of foodstuff production for *Enterobactericeae*: an average of 9% for organic and 11.9% for isolates recovered from conventionally produced items. Globally, values of nonsusceptibility for the two quinolones tested ranged between 0% and 4.9% for conventional produce (Table 8.2). Nonsusceptibility values for *Enterobactericeae* against aminoglycosides (0.5% to 3.8%), chloramphenicol (3.8%) and sulfonamides (0.5% to 1.1%) also remained low, showing no significant differences between isolates recovered from organic and conventional fruits and vegetables (Table 8.2 and Table S8.1).

For *Acinetobacter* spp., (n=89) nonsusceptibility to β-lactam antibiotics ranged between imipenem/cefepime (0%) for both types of production, and cefotaxime (63.0%) for isolates recovered from conventionally produced fruits and vegetables. Nonsusceptibility to other assessed antibiotics (ciprofloxacin, gentamicin, amikacin and tetracycline) showed 0 to 1.1% of nonsusceptibility in bacteria from this genus recovered from conventional and organic production, respectively (Table 8.2).

When *Pseudomonas* spp. (n=35) isolates were analyzed, low levels of antibiotic nonsusceptibility were detected. Globally, the maximum and minimum values fluctuated between 0% for ceftazidime/ciprofloxacin and ciprofloxacin/gentamicin/amikacin for organic and conventional produce, respectively, and 8.6% for ceftazidime/imipenem for isolates collected from conventionally produced items (Table 8.2).

Stenotrophomonas malthophilia isolates (n=13) recovered from lettuces (n=11), carrots (=1) and tomatoes (n=1) were all susceptible to trimethoprim/sulfamethoxazole, which is the only agent for which EUCAST breakpoints are currently available (see legend Table 8.2).

Overall, *E. coli* (n=2), *M. morganii* (n=1), *Acinetobacter* spp. (n=1), *Enterobacter* spp. (n=1) and *K. pneumoniae* (n=1) showed multidrug resistance, i.e. nonsusceptibility to three or more structurally unrelated classes of antibiotics (Magiorakos et al., 2011).

Synergy tests identified presumptive phenotypic production of ESBL, AmpC β-lactamases, MBL, penicillinases and co-production of those β-lactamases (Figure S8.1). Their detection was associated with the origin of the samples to determine that 60.3% and 32% of the isolates recovered from conventionally and organically produced fruits and vegetables, respectively, were presumptive AmpC producers; 0.8% isolates from conventionally produced items were identified as phenotypically positive for ESBL production by the presence of synergy between cefotaxime and clavulanic acid, ceftazidime and clavulanic acid or both (Figure S8.1).

Table 8.2. Percentage of isolates from organic and conventionally produced fruits and vegetables nonsusceptible to antibiotics according with their family (n=308).[a]

Antibiotic	*Enterobactericeae* (n=184)		*Acinetobacter* spp. (n=89)		*Pseudomonas* spp. (n= 35)	
	Org	Conv	Org	Conv	Org	Conv
β-lactams						
Ampicillin	29.3	45.1	NA	NA	NA	NA
Cefotaxime	6.5	5.4	31.5[b]	63.0[b]	NA	NA
Ceftazidime	5.4	5.4	6.7[b]	13.5[b]	0	8.6
Cefepime	0.0	0.0	0[b]	0[b]	2.9	5.7
Cefoxitin	19.6	26.6	NA	NA	NA	NA
Imipenem	0.0	0.0	1.1	0	2.9	8.6
Ertapenem	2.2	1.1	NA	NA	NA	NA
Quinolones						
Nalidixic acid	3.3	4.9	NA	NA	NA	NA
Ciprofloxacin	1.1	0.0	1.1	0	0	0
Aminoglycosides						
Gentamicin	1.1	3.8	1.1	0	2.9	0
Amikacin	0.5	0.5	1.1	0	2.9	0
Tetracyclines						
Tetracycline	NA	NA	1.1[b]	0[b]	NA	NA
Phenicols						
Chloramphenicol	3.8	3.8	NA	NA	NA	NA
Sulfonamides						
Trimethoprim/ Sulfamethoxazole	1.1	0.5	NA	NA	NA	NA

[a] *Aeromonas* spp., *Comamonas* spp. and *Delftia* spp. (n=12) were not included due to the inexistence of breakpoints to interpret nonsusceptibility results; isolates from genera *Stenotrophomonas* (n=13) were all susceptible to sulfamethoxazole/trimethoprim, which corresponds to the only EUCAST or CR-SFM available;
[b] CR-SFM breakpoints were applied due to inexistence of EUCAST breakpoints;
NA, No EUCAST or CR-SFM breakpoints were available for the antibiotic.
Org, organic; Conv, conventional.

Overall, 80.1% of the isolates produced a penicillinase. MBL were produced by 1.6% of the isolates, distributed among conventional and organic recovered isolates grown exclusively on the soil (Figure S8.1).

Odds ratios were used to compare the relative probability of the occurrence of antibiotic nonsusceptible isolates, given exposure to different modes of production (i.e. organic and conventional) and level of growth (i.e. in, on and above the soil). This analysis identified variables associated with nonsusceptibility to different antibiotics (ceftazidime, cefepime, imipenem, ciprofloxacin and gentamicin) and presence of multidrug resistance (Table S8.1). Although protective associations have been noticed for a set of different associations (e.g. ceftazidime for organic items from all types of soil growth taken together) these correlations were not found to be statistically significant (Table S8.1).

Genomic characterization of integron-harboring isolates

Overall, among the 333 isolates studied, we have detected eleven isolates harboring twelve integrons, as follows: *E. cloacae* (n=2), *E. coli* (n=4), *R. ornithinolytica* (n=2), *M. morganii* (n=1), *P. putida* (n=1) and *K. pneumoniae* (n=1) (Table 8.3). A single *K. pneumoniae* isolate harboured a class 1 and a class 3 integron within its genome. Integrons were similarly recovered along the whole period of sampling (March 2013 to February 2014), distributed among organic (n=6) and conventional (n=5) food products, and were predominant among lettuces (n=9), but were also present in carrots (n=1) and strawberries (n=1) (Table 8.3).

The genome sequences of the integrase-positive isolates were subjected to bioinformatics analyses. The main statistics obtained with the *de novo* assembly of the eleven genomes are displayed in Table S8.2. The draft genomes varied among 3.8 Mb and 3688 protein-coding genes for *M. morganii*, and 6.5 Mb and 5938 protein-coding genes for *P. putida*, respectively (Table S8.2).

Besides the presence of typically chromosomal resistance genes such as $bla_{\text{ACT-type}}$ and $bla_{\text{DHA-type}}$ in *E. cloacae* and *M. morganii* (data now shown), respectively, 10 out of the 11 isolates showed acquired resistance genes: INSali2 (*aadA1b, bla*$_{TEM-1}$*, dfrA1b, sul1, tetA*), INSali10 (*aadA2, aadB, qnrA1, sul1*), INSali25 (*aadA1y, aph(4)-Ia,* $bla_{\text{TEM-1}}$, *estX-12, floR*, *mcr-1*, *strA, strB, sat2 sul2, tetA*), INSali38 (*aac(3)-Via, aadA1a,* $bla_{\text{TEM-1}}$, *strA, strB, sul1, sul2, tetA*), INSali92 (*aadA1b,* $bla_{\text{TEM-1}}$, *catA1, dfrA1b, strA, strB, sul1, sul2, tetA*), INSali127 and INSali133 (*aadA1y, sat2*), INSali207 (*aadA1a, aadB, cmlA1d, mphA, sul1, tetB*), INSali370 (*aadA2, bla*$_{TEM-1}$*, dfrA12, strA, strB, sul1, sul2, tetA*) and INSali390 (*aadA2br,* $bla_{\text{GES-11}}$, $bla_{\text{SHV-28}}$, *dfrA12, dfrB3, oqxAB, sul1, tetD*). Together, the acquired resistance genes identified could guarantee resistance to multiple antibiotic classes, such as β-lactams, aminoglycosides, quinolones, chloramphenicol and sulfonamides, as represented in the susceptibility profile displayed in Table 8.3. Among the acquired resistance mechanisms, we highlight the presence of the recently described plasmid-mediated colistin resistance gene *mcr-1* in *E. coli*, the plasmid-mediated quinolone resistance (PMQR) gene *qnrA1* in *E. cloacae*, and the macrolide inactivation gene *mphA* in *M. morganii,* all from lettuce samples, as well as ESBL-encoding gene $bla_{\text{GES-11}}$ and PMQR-encoding gene *oqxAB* in *K. pneumoniae* recovered from organic strawberries.

The assessment of STs, showed that one of the *E. cloacae* and one of the *E. coli* isolates were assigned to new allele combinations, that were registered in the respective MLST databases as ST636 and ST5981 (Table 8.3). In addition, other STs were detected for the

remaining typable species: *E. cloacae* (ST90), *E. coli* (ST345, ST1716 and ST2522) and *K. pneumoniae* (ST15) (Table 8.3).

According with PlasmidFinder (>98% identity), the majority of the isolates harboured multiple known *Enterobactericeae* plasmids from the following incompatibility groups: INSali2 (IncX), INSali10 (IncFIB, IncFII), INSali25 (IncHI2, IncI1, IncI2, IncQ, IncP, IncY), INSali38 (IncHI2, IncFIC, IncP, IncQ), INSali92 (IncHI2, IncP, IncQ), INSali127 and INSali133 (IncL/M), INSali370 (IncQ), and INSali390 (IncFIB, IncQ, IncR, IncFII). Globally, 1 to 6 intact prophage regions were detected within the genomes of these Gram negative isolates. For *E. coli* isolates INSali25, INSali38, INSali92 and INSali370, virulence factors *gad*, *lpfA*, *capU* were differently detected, as displayed in Table 8.3.

According with PathogenFinder, the majority of the isolates showed a significant possibility of acting as human pathogens: the values have levelled off between 66.6% for *M. morganii* INSali207 and 94.2% for the clinically relevant *E. coli* isolates INSali38 and INSali92*. P. putida* INSali382 was the only integron-positive isolate predicted as a non-human pathogen (8.8%), showing also no acquired antibiotic resistance genes (Table 8.3).

Diversity of mobile resistance integrons

Among the eight class 1 integrons analyzed, two were assigned to new integron numbers due to the detection of novel gene cassette arrays.

The 12,038 bp sequence of isolate *E. cloacae* INSali2 (Figure 8.2A) was assigned to In*369,* and enclosed an array containing gene cassettes *drfA1b* and *aadA1b* and respective *attC* sites, encoding resistance to trimethoprim and aminoglycosides, respectively. This Tn*402*-like integron contained a complete 5'CS (common segment) encompassing *intI1* gene and respective promoter (P*intI1*), gene cassette promoter (Pc) variant PcW and *attI1* integration site. The 3'CS was composed by *qacΔE1*, *sul1* and *orf5* genes, followed by a complete Tn*2**. The incomplete transposition module of Tn*402* was detected downstream of the 3'CS region (*tniAB*), and the initial and terminal inverted repeats (IRi and IRt) were also present in both extremities.

The region enclosing complex integron In*293*::IS*CR1*::*qnrA1*, which was recovered from the genome of *E. cloacae* INSali10 isolate is represented in Figure 8.2B, reaching up to 10,509 bp. The upstream region of this integron showed a complete *intI1*, P*intI1*, PcH1 and *attI1* integration site. The isolate showed a variable region 1 (vr-1) comprised of *aadB* and *aadA2* genes, which encode resistance to different antibiotics of the aminoglycosides class, followed by respective *attC* sites, and a first 3'CS composed of *qacΔE1* and *sul1*.

Table 8.3. Phenotypic and genotypic characteristics of isolates producing class 1, 2 and 3 integrons.

Isolate	Sample	Date	Type of production	Produce	NS profile	Acquired antibiotic resistance genes	Virulence factors	Human pathogen probability (%)[a]	MLST	Plasmids	Number of phage regions	Integron classes
E. cloacae												
INSali2	VG2	March 2013	Conventional	Lettuce	A, Ac, F, S	*aadA1b*, *bla*$_{TEM-1}$, *dfrA1b*, *sul1*, *tetA*	-	75.2	ST636	IncX	2	1
INSali10	VG7	March 2013	Organic	Lettuce	A, Ac, Cr, F, G	*aadA2*, *aadB*, *qnrA1*, *sul1*	-	75.6	ST90	IncFIB, IncFII	2	1
E. coli												
INSali25	VG26	May 2013	Conventional	Lettuce	A, N, G, C	*aadA1y*, *aph(4)-Ia*, *bla*$_{TEM-1}$, *estX-12*, *floR*, *mcr-1*, *sat2*, *strA*, *strB*, *sul2*, *tetA*	*gad*	93.4	ST1716	IncHI2, IncI1, IncI2, IncQ, IncP, IncY	6	2
INSali38	VG37	June 2013	Conventional	Lettuce	A, G	*aac(3)-Via*, *aadA1a*, *bla*$_{TEM-1}$, *strA*, *strB*, *sul1*, *sul2*, *tetA*	*lpfA*, *CrpU*	94.2	ST5981	IncHI2, IncFIC, IncP, IncQ	3	1
INSali92	VG85	October 2013	Organic	Lettuce	A, C	*aadA1b*, *bla*$_{TEM-1}$, *CrtA1*, *dfrA1b*, *strA*, *strB*, *sul1*, *sul2*, *tetA*	*lpfA*, *CrpU*	94.2	ST2522	IncHI2, IncP, IncQ	2	1
INSali 370	VG85	October 2013	Organic	Lettuce	A, N, Ci, S	*aadA2*, *bla*$_{TEM-1}$, *dfrA12*, *strA*, *strB*, *sul1*, *sul2*, *tetA*	*lpfA*	94.0	ST345	IncQ	5	1
R. ornithinolytica												

INSali127	VG131	January 2014	Organic	Carrot	A	*aadA1y, sat2*	-	83.2	NA	IncL/M	4	2
INSali133	VG128	January 2014	Organic	Lettuce	A	*aadA1y, sat2*	-	83.1	NA	IncL/M	4	2
M. morganii												
INSali207	VG26	May 2014	Conventional	Lettuce	A, Ac, N, G, C	*aadA1a, aadB, cmlA1d, mphA, sul1, tetB*	-	66.6	NA	-	3	1
P. putida												
INSali382	VG129	January 2014	Organic	Lettuce	-	-	-	8.8	NA	-	1	1
K. pneumoniae												
INSLA390	VG136	February 2014	Organic	Strawberry	A, Ac, Ct, Cr, N, Ci, S	*aadA2br, bla*$_{\text{GES-11}}$, *bla*$_{\text{SHV-28}}$, *dfrA12, dfrB3, oqxAB, sul1 tetD*	-	87.8	ST15	IncFIB, IncQ, IncR, IncFII	4	1, 3

[a] Probability of isolate being a human pathogen according with Pathogen Finder (www.cge.cbs.dtu.dk/services/PathogenFinder);
A, ampicillin; Ac, amoxicillin with clavulanic acid; Ct, cefotaxime; Cr, ceftazidime; F, cefoxitin; N, nalidixic acid; Ci, ciprofloxacin; G, gentamicin; C, chloramphenicol; S, trimethoprim/sulfamethoxazole;
NS, nonsusceptibility.
MLST, Multilocus sequence typing;
NA, Not applicable.

This region was pursued by an IS*CR1* (insertion sequence common region 1). Variable region 2 (vr-2) included a *qnrA1*, an *ampR* and a *ΔhybF*, which encode a zinc-containing protein associated with NiFe hydrogenase. This second array was followed by the second 3'CS, which is characteristic of complex integrons backbones, and an additional open reading frame (*orf6*). Although *tniAB* module was absent, Tn*402* IRi and IRt were still detected (Figure 8.2B).

The Tn*402*-like class 1 integron recovered from *E. coli* INSali38 (Figure 8.2C) exhibited an *intI1* interrupted by the insertion of a cluster of resistance genes (*sul2-strA-strB*), which was preceded by genes involved in plasmid replication (*repAC*) (data not shown). Nevertheless, P*intI1*, promoter PcW, a second cassette promoter P2 and *attI1* were present. Following the 5'CS, the integron variable region enclosed only one gene cassette with an identifiable open reading frame and attendant *attC* site: *aadA1a*. But the gene cassette was then followed by an array of genes including aminoglycoside resistance gene *aac(3)-VIa*, *groES* and *groEL*, which encode co-chaperone GroES and chaperone GroEL, and *insE* and *insF* genes, typically encoding IS*3* transposase InsEF. Afterwards, this 20,220 bp length region enclosing In*2* harboured a conventional *qacΔE1-sul1-orf5* 3'CS that was then followed by IS*1326* (IS*21* family) and by a Tn*402*-like *tniAB* transposition module, as well as IRt.

Similarly to INSali38, 11,395 bp Tn*402*-like integron harboured by *E. coli* INSali92 was preceded by *repAC* and *sul2-strA-strB* gene cluster, interrupting the beginning of *intI1* (Figure 8.2D). In this case, PintI1, PcW and *attI1* were also detected. The gene cassette array of this In*369*, which was identical to INSali2, included *drfA1b* and *aadA1b* antibiotic resistance genes with their respective *attC* sites. Then, it was followed by *qacΔE1-sul1-orf5* and *tniAB* transposition module. The 3' terminus was delimited by Tn*402* IRt.

In*1259*, firstly detected *in M. morganii* INSali207, represents a typical class 1 integron. However, IRi was not present and *intI1* was truncated by the insertion of an IS*26* transposase (Figure 8.2E). The P*intI1* and *attI1* were detected, and so was promoter PcW showing an extended -10 motif (TNG) (PcW_{TNG-10}). The variable gene cassette array that encoded *aadB*, *aadA1a* and *cmlA1d* was followed by the 3'-CS gene cluster *qacΔE1-sul1* and by a *Δorf5. T*he downstream flanking region was composed by an IS*6100, a* chromate transporter-encoding gene *chrA*, and by macrolide inactivation gene cluster *mphA-mrx-mphR* outside the boundaries of the integron (Figure 8.2E).

E. coli INSali370 harboured In*27*, enclosing *dfrA12*, *gcuF* and *aadA2* as well as the correspondent *attC* locations in the variable region (Figure 8.2F). This Tn*402*-like integron was limited by *intI1* gene, P*intI1*, PcW_{TNG-10} and *attI1* recombination site at 5' end, while the conserved 3'CS was comprised of *qacΔE1*, *sul1* and *orf5*. 3'CS was also limited by the

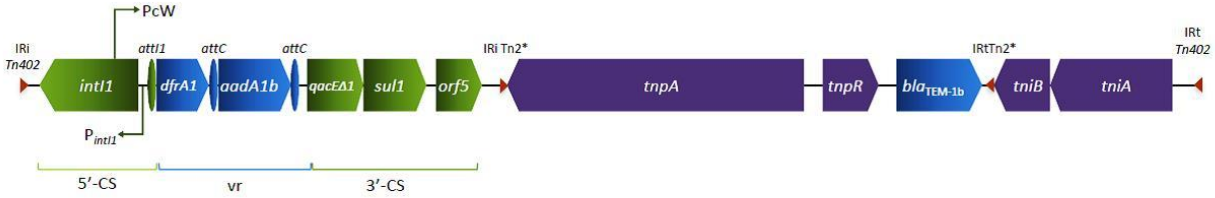
incomplete transposition module of Tn*402* (*tniAB*) but the IRt was missing, unlike IRi at the 5'CS. Similarly, *K. pneumoniae* INSali390 harboured a class 1 integron that was identical to In*27* along the entire array. However, this isolate showed the presence of a PcS promoter and *aadA2* was substituted by the novel derivative *aadA2br*, which changed In*27* into the new In*1258* (Figure 8.2G).

The class 1 integron present in the genome of *P. putida* INSali382 contained a variable region for which no significant homology could be found in existing integrons (Figure 8.2H). In this In*0*, gene cassettes for known antibiotic resistance determinants were not present and neither the traditional 3'CS region encoding *qacΔE1*, *sul1* and *orf5*. This Tn*402*-like integron was limited upstream by a complete *intI1*, P*intI1*, PcW and *attI1*, and downstream by a complete Tn*402* transposition module – *tni*ABQR – which was followed by a *mer* operon, encoding resistance to mercury. IRi and IRt enclosed this atypical class 1 integron.

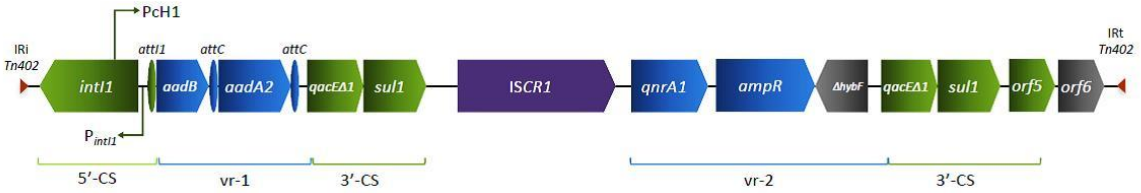
Different class 2 integrons were detected in *E. coli* INSali25 (In*2-35*) and in *R. ornithinolytica* INSali127 and INSali133 isolates (In*2-11*) (Figure 8.3A-C). Just like the majority of class 2 integrons, these two integrons were flanked by a truncated *IntI2*, respective promoter P*intI2*, Pc and *attI2* recombination site in the upstream region of the integron. The downstream region was comprised of the complete Tn*7* transposition module and the typical hypothetical proteins-encoding *ybeA*, *ybfA*, *ybfB* and *ybgA* (Figure 8.3A, B and C). The gene cassette arrays, which were followed by the respective *attC* sites, included genes coding for resistance to aminoglycosides and streptothricin, as follows: *estX12-sat2-aadA1y* for *E. coli* INSali25 and *sat2-aadA1y* for *R. ornithinolytica* INSali127 and INSali133. IRi and IRt were detected in all three integrons.

The new class 3 integron In*3-13* was detected in *K. pneumoniae* INSali390 isolate (Figure 8.4) that also carried the new In*1258* (Figure 8.2G). The 8873 bp length region enclosing the class 3 integron was comprised of an upstream region encoding complete *intI3,* P*intI3*, Pc variant 1 and *attI3*. This region showed 100% identity with In*3-9*, whose sequence was recently submitted to Genbank (KT984195), after being detected in *Citrobacter freundii* from a hospital effluent in France. The variable gene array enclosed the ESBL-encoding gene bla_{GES-11}, which was followed by the dihydrofolate reductase type B-encoding gene *dfrB3* and the respective *attC* recombination sites. The array of acquired antibiotic resistance genes was followed by genes encoding proteins essential for plasmid replication (*repC, repA)* and mobilization (*mobC, mobA*). No transposons were detected in the regions immediately adjacent to In*3-13* (Figure 8.4).

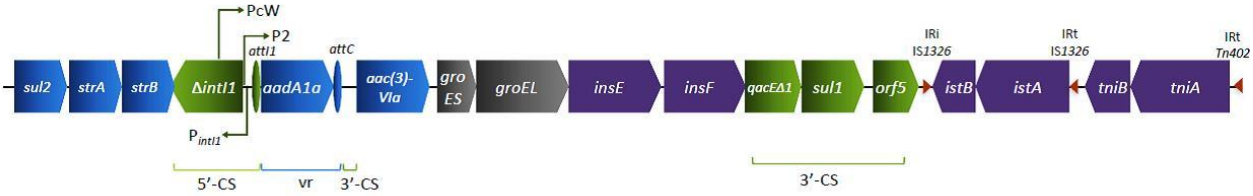
A) *E. cloacae* INSali2 In*369*



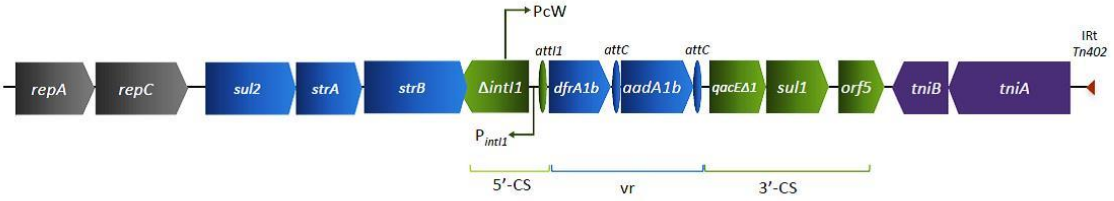
B) *E. cloacae* INSali10 In*293*::IS*CR1*::*qnrA1*



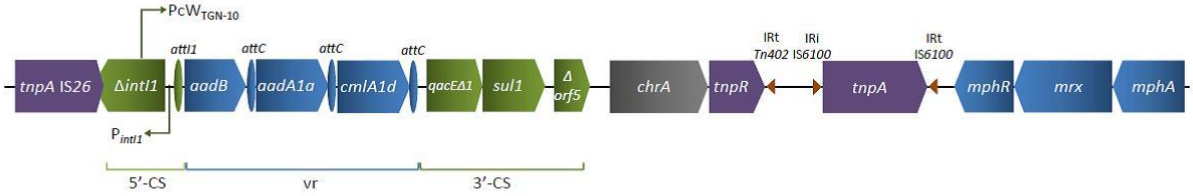
C) *E. coli* INSali38 In*2*



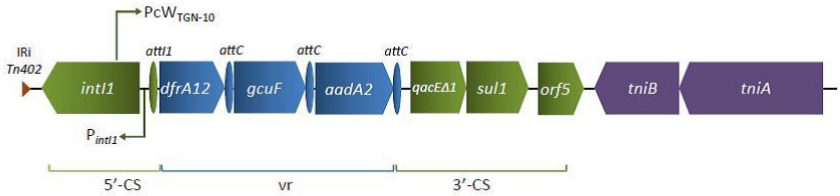
D) *E. coli* INSali92 In*369*



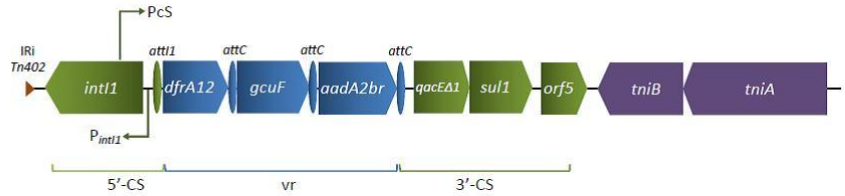
E) *M. morgannii* INSali207 In*1259*



F) *E. coli* INSali370 In*27*



G) *K. pneumoniae* INSali390 In*1258*



H) *P. putida* INSali382 In*0*

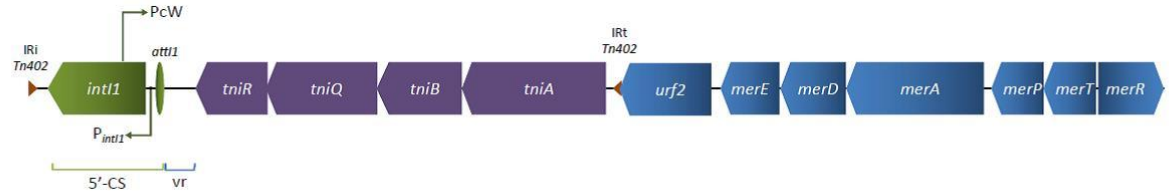


Figure 8.2. Schematic representation of regions enclosing class 1 integrons detected among the bacterial population analyzed in the present survey (n=8/333). A) *E. cloacae* INSali2 In*369;* B) *E. cloacae* INSali10 In*293*::IS*CR1*::*qnrA1;* C) *E. coli* INSali38 In*2;* D) *E. coli* INSali92 In*369;* E) *M. morganii* INSali207 In*1259*; F) *E. coli* INSali370 *In27*; G) *K. pneumoniae* INSali390 In*1258*; H) *P. putida* INSali382 In*0*. Green, integron; blue, resistance genes, including gene cassettes; purple, transposons; grey, other genes.

8.5. Discussion

The current study focused on evaluating organically and conventionally grown produce with regard to antibiotic nonsusceptibility and integron content to understand their contribution for the spread of antibiotic resistance in a specific division of the food chain. Overall, it led to three main global findings. First, Gram negative bacteria were frequently isolated from raw and unwashed fruits and vegetables. Among the 144 samples from fresh produce, 78 samples carried this type of bacteria, among which, exactly 39 were conventionally and 39 were organically produced. Moreover, no statistically significant correlation was found between the presence of resistance to specific antibiotics and the way in which the food products were produced (Table S8.1), as also previously reported (Ruimy et al., 2010).

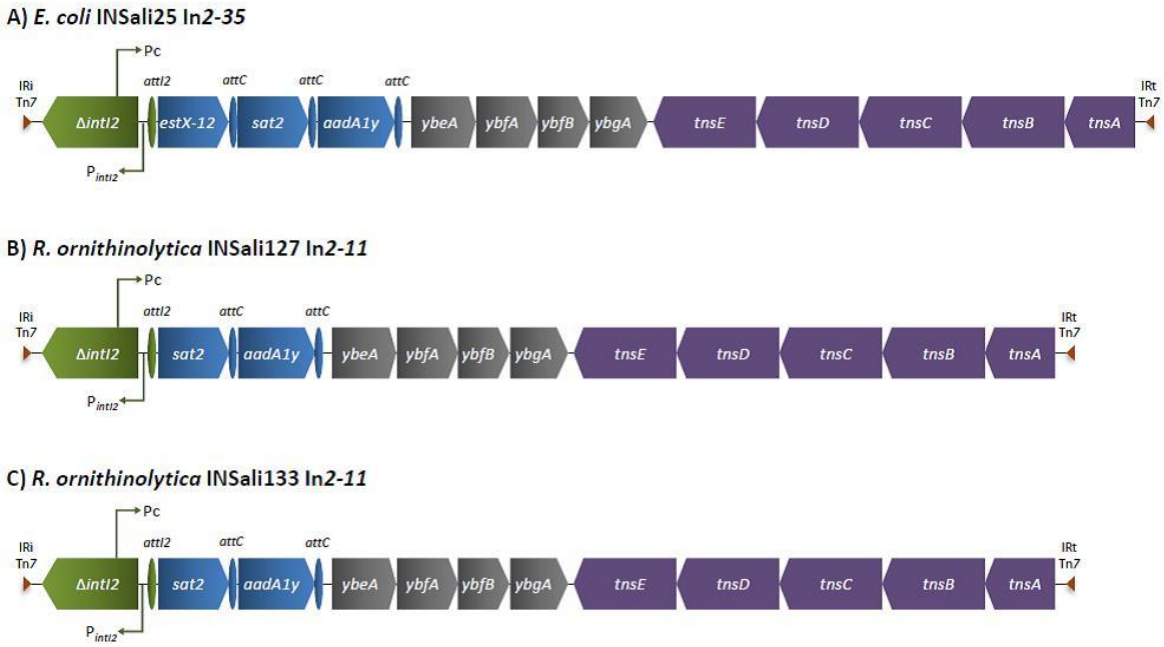


Figure 8.3. Schematic representation of regions enclosing class 2 integrons detected among the bacterial population analyzed in the present survey (n=3/333). A) *E. coli* INSali25 In*2-35*; B) *R. ornithinolytica* INSali127 In*2-11*; C) *R. ornithinolytica* INSali133 In*2-11*. Green, integron; blue, resistance genes, including gene cassettes; purple, transposons; grey, other genes.

Although it has been formerly suggested that some agricultural practices may constitute a risk for the emergence of resistance to specific antibiotics (Hartmann et al., 2012; Jones-Dias et al., 2016), in this study, different agricultural practices did not translate into different amounts of risk. Second, comparison with other reports of nonsusceptible Gram negative bacteria recovered from specific retail vegetables revealed that this study found lower levels of resistance, namely to third generation cephalosporins in *Enterobactericeae*,

suggestive of ESBL production (Kim et al., 2015; van Hoek et al., 2015). In fact, we have only detected six multidrug resistant isolates. The third main conclusion was that certain organic and conventional products carried isolates harboring class 1, 2 and/or 3 integrons that also harboured clinically relevant acquired antibiotic resistance genes.

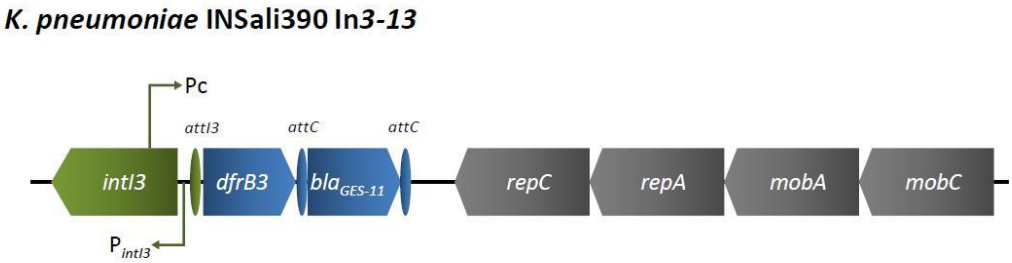


Figure 8.4. Schematic representation of region enclosing In*3-13* detected in a *K. pneumoniae* recovered among the bacterial population analyzed in the present survey (n=1/333). Green, integron; blue, resistance genes, including gene cassettes; grey, other genes.

Enterobacter spp. is often described as intestinal commensal of humans and animals and frequently isolated from cases of hospital- and community-acquired urinary tract infections in immunocompetent and immunocompromised hosts (Chang et al., 2013).
In this study, this genera was not only the second most common Gram negative bacteria isolated, but *E. cloacae* was also among integron producers. Other important *Enterobactericeae* human commensals, such as *E. coli*, *M. morganii*, *Klebsiella* spp., *Serratia* spp. and *Providencia* spp. may also act as pathogenic agents and were also detected in this study to a less extent (Marshall et al., 2009). *Pseudomonas* spp. and particularly *Acinetobacter* spp., which are primarily pathogenic for immunocompromised hosts, were the first and third most frequently detected genera. In this study, no strict pathogenic agents were detected. However, our search was only focused in antibiotic resistance bacteria, which could have limited the appearance of antibiotic susceptible pathogens. The conclusions about the influence of vegetables in the ecology of the human microflora are diverse. In fact, there is evidence that the prevalence of antibiotic resistant bacteria in the gastrointestinal flora may vary with dietary habits (van Den Braak et al., 2001). Our results suggest that the microbial content of unwashed and unpeeled fruits and vegetables may constitute a threat for the immunocompromised population, as corroborated by other authors (Marshall et al., 2009; Losio et al., 2015). In Europe, reports of antibiotic resistance bacteria in food products imported from other continents is frequent (Wang et al., 2011; Zurfluh et al., 2015a; Zurfluh et al., 2015b; Zurfluh et al., 2016). However, samples were obtained from products grown and marketed in Portugal, suggesting that

international food trade isn't always a requirement for contamination of fresh produce with antibiotic resistance pathogenic agents.

The genomic characterization of isolates harboring class 1, 2 and 3 integrons showed that these mobile genetic elements were mostly found among commensal bacteria and opportunistic pathogens. In fact, the probability of human pathogenicity, estimated upon the detection of known *γ-proteobacteria* pathogenicity factors, showed that only *P. putida* INSali382 was not a probable human pathogen. *R. ornithinolytica* is not often associated with human infections (Khajuria et al., 2013; Chun et al., 2015). In this study, this species was isolated from organic samples of lettuce and carrot collected at the same retail store. Indeed, the genetic similarity between isolates INSali127 and INSali133 suggests the contamination of both products by a single source. Similarly, *E. coli* isolates ST2522 INSLA92 and ST345 INSLA370 were recovered from the same lettuce sample, representing an example of contamination of a unique produce with multiple antibiotic resistance microorganisms. Moreover, multidrug resistant ST15 *K. pneumoniae*, which in this study was detected in organically-produced strawberries, represents a major clone among nosocomial infections, as previously reported (Hu et al., 2013; Manageiro et al., 2015a; Markovska et al., 2015). Globally, our microbiological results corroborate the statements of Berg et al. (2014) reporting that large scale production and distribution of vegetables, together with an increasing number of immunocompromised individuals, may result in an enhanced number of human infections associated with consumption of vegetables.

When we analyzed the acquired resistance mechanisms from the eleven integron-producing isolates, some specific genes hold our attention. The identification of the *mcr-1* gene in an *E. coli* recovered from a conventionally produced lettuce, confirmed that the food chain is deeply involved in the dissemination dynamic of acquired resistance genes (Hasman et al., 2015; Liu et al., 2016; Stoesser et al., 2016; Yao et al., 2016). Considering that colistin is a last resource antibiotic, used for the treatment of infections caused by multidrug resistant bacteria, the detection of a mobile colistin resistance gene in a raw vegetable constitutes a serious and unprecedented public health concern (Paterson and Harris, 2016). Although unusual, the presence of the *mphA* gene cluster in *M. morganii* is of little clinical significance, because *Enterobactericeae* are intrinsically resistant to macrolides due to the presence of efflux transporters (Leclercq, 2002; Poole et al., 2006). However, considering that the human gut is prone to the occurrence of increased horizontal gene transfer between bacteria, *mphA* gene might get transferred to commensal Gram positive bacteria (Stecher et al., 2012); this and the supplementary array of plasmids and prophages harboured by integrons-positive isolates reinforced the potential

concerted contribution of different mobile genetic elements to the mobilization and spread of acquired resistance (Fernandez-Lopez and de la Cruz, 2014).

We choose to mainly focus the investigation on the elements most likely associated with the dissemination of acquired resistance in the food chain. The identification of class 1 (n=8), class 2 (n=3) and class 3 (n=1) integrons has led to the identification of many gene cassettes and diversity of integrons backbones.

In this study, Tn*402*-like class 1 integrons were identified in isolates INSali2, INSali 10, INSali38, INSali92, INSali370, INSali382 and INSali390. This transposon is bound by two inverted repeats (IRi and IRt) and acts as the main carrier element for class 1 integrons, which usually display an incomplete transposition module composed of two *tni* open reading frames (Gillings et al., 2009; Sajjad et al., 2011). Notably, class 1 integrons showed an impressive mesh of interactions with several other transposable elements (such as Tn*402*, *ISCR*1, Tn*2**, IS*26*, IS*1326* and IS*3*), also revealing close regions that denote their presence in resistance plasmids (*rep* and *mob* genes). Important evolutionary steps shaped the basic class 1 integron backbone that now dominates clinical and non clinical bacterial isolates. The first was probably the insertion of a class 1 integron into the a Tn*402* transposon, as also shown in Figure 8.2; the second step comprised the creation of the 3'-CS, which involved acquisition of *qacE1*, *sul1* and *orf5*, followed by the loss of part of *qacE1* gene and part of the Tn*402* transposition module, as shown in Figure 8.2 (Gillings et al., 2009). The In*0* described in isolate *P. putida* INSali382 appears to be a descendant of these original events, representing a key intermediate in the evolution of the clinical class 1 integron. Moreover, this integron showed unusual 3'CS context, displaying active transposition machinery, which enables greater mobility. With these integron/transposable elements data taken together, it is clear that functional integrons/transposons are well established in the bacterial population, either from human (e. g. *K. pneumoniae*) or environmental origin (e.g. *P. putida*), contributing actively to the movement of resistance genes. Apart from the examples reported here in fresh produce bacterial population, other recent examples with class 1 integrons linked to functional Tn*402* modules include bacteria from other food items (Sajjad et al., 2011), clinical samples (Chen et al., 2014b) and the environnent (Rosewarne et al., 2010). Collectively, these integrons/transposons demonstrate dynamic exchange of cassettes, given the diverse combinations of cassettes observed, among which *aadA* and *drfA* were predominant. Overall, Pc variants with different strengths were also detected in the class 1 integrons, based on the sequence of the −35 and −10 elements. From the eight variants initially described, we detected weak promoter PcW (n=4), hybrid promoter PcH1 (n=1), and the stronger variants PcW_{TNG-10} (n=2) and PcS (n=1) and the additional P2 (n=1).

Although the predominant PcW is described as the weakest promoter of all, it is also associated with higher integrase excision activity, compensating the lower expression levels of the variable gene array with an increased ability to incorporate gene cassettes (Vinué et al., 2011).

The class 2 integrons detected in one *E. coli* and in two genetic related R. *ornithinolytica isolates* corresponded to In*2-35* and In*2-11*, already described in *E. coli* from animals and *V. cholerae* O1 clinical strain (Kadlec and Schwarz, 2008; Sá et al., 2010). As noted in this study, class 2 integrons normally show less variability than class 1 and 3 integrons, due to the lack of integration activity of *intI2* gene, which is commonly truncated. The frequent persistence of genes such as *estX* and *sat* genes in a considerable number of class 2 integrons, suggests co-selection of these genes even in the absence of a direct selective pressure. In fact, streptothricin antibiotics have not been used as therapeutics, but for a long time this antibiotic has been used as growth promoters in veterinary, which justifies their current widespread distribution (Looft et al., 2012). Overall, the gene cassettes detected within the variable region of class 2 integrons provide a smaller contribution to multidrug resistance phenotypes, than those of class 1 integrons (Ramírez et al., 2010).

The variable region of the new class 3 integron (In*3-13*) detected in *K. pneumoniae* INSali390 exhibited a *dfrB3* and a $bla_{\text{GES-11}}$. GES-containing class 3 integrons have already been described. Curiously, two of them, In*3-2* [$bla_{\text{GES-1}}$, $bla_{\text{OXA-10}}$/*aac(6')-Ib*] and In*3-8* ($bla_{\text{IMP-8}}$, $bla_{\text{GES-5}}$, $bla_{\text{BEL-1}}$, *aacA4*), were described in *K. pneumoniae* from Portuguese health care settings in 2003 and 2015, respectively (Correia et al., 2003; Papagiannitsis et al., 2015). Considering this information, the report of a GES-containing class 3 integron in a pathogenic *K. pneumoniae* suggests food contamination from clinical sources. At the boundaries of the In*3-13* we detected genes associated with plasmid replication (*repA*, *repC*) and mobility (*mobA*, *mobC*), confirming its transference potential. This corroborates results from other class 3 integron-containing plasmids that have showed the presence of *rep* genes downstream of the gene cassettes (Papagiannitsis et al., 2015). Although class 3 integrons have been associated with clinical and environmental samples, to the best of our knowledge, this study constitutes the first assigned class 3 integron from food products of vegetable origin (http://integrall.bio.ua.pt/). Overall, the pathogenic ST15 *K. pneumoniae* producer of new In*1258* and In*3-13* recovered from organic strawberries constitute a food safety concern.

Taken together, our results fully support the growing concern about the presence of mobile resistance genes in commensals and/or potential pathogens found in vegetable microbiomes (Berg et al., 2014). The detection of integrons of three different classes, associated with other clinically relevant mobile genetic elements and acquired antibiotic

resistance genes, such as the new *mcr-1* gene, reinforce the mobilization potential of antibiotic resistance in Gram negative bacteria. Although continuous monitoring of products of vegetable and animal origin is essential, the decrease or cessation of certain antibiotics in agriculture is strongly advised to preserve drug molecules for clinical treatment of infections.

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Chapter 9.

First description of plasmid-mediated colistin resistance (mcr-1) in Escherichia coli from non-imported fresh vegetables in Portugal

This research paper was submitted as:

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Contributions of the authors for the following manuscript:

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Vera Manageiro: analysis of data, critical revision of the manuscript, final approval of manuscript;*
Luís Vieira: acquisition of laboratory data, final approval of manuscript;
Eugénia Ferreira: acquisition of laboratory data, critical revision of the manuscript, final approval of manuscript;
Manuela Caniça: conception and design of study, critical revision of the manuscript, final approval of manuscript.

** these authors contributed equally to this work.*

9.1. Abstract

In this study, the authors report the detection of the new plasmid-mediated colistin resistance *mcr-1* gene in an *Escherichia coli* recovered from a non-imported lettuce, produced and marketed in Portugal, and acquired at a hypermarket, indicating the widespread presence of the gene in the food chain. Moreover, the gene showed a different genetic environment from others previously reported. The spread of this gene constitutes a severe and unprecedented concern in the field of human infectious diseases.

9.2. Main text

Fresh produce has been increasingly implicated in bacterial outbreaks. Indeed, fruits and vegetables can become contaminated with antibiotic resistance bacteria before wholesale distribution, through amendments with manure or untreated wastewater (Berg et al., 2014). This poses a serious health risk to humans, which may result in sporadic cases of gastroenteritis, outbreaks and permanent colonization (Berger et al., 2010). Moreover, currently, colistin constitutes one of the few therapeutic options available for the treatment of infectious diseases caused by multidrug resistant *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Gaibani et al., 2014; Ly et al., 2015). However, colistin resistance has been emerging in humans and animals, and intra-species transmission of resistant isolates has already been reported (Kieffer et al., 2015; Olaitan et al., 2015; Bathoorn et al., 2016). It is crucial to preserve this antibiotic and carefully monitor the dissemination of the recently identified plasmid-mediated colistin resistance gene *mcr-1*. The detection of mobile colistin resistance determinants in the food chain underscores the mobility potential involved in this resistance and raises significant human food safety concerns (Liu et al., 2016).

In the scope of the analysis of the antibiotic nonsusceptibility of a collection of Gram negative isolates recovered from fresh fruits and vegetables, produced and marketed in Portugal, we characterized 10 isolates harboring integrons that were subjected to whole genome sequencing (Myseq, Illumina, San Diego, CA), according to what is described elsewhere (Manageiro et al., 2015c). We identified the presence of the plasmid-mediated colistin resistance gene *mcr-1* in an *E. coli* isolate, which had been recovered from a lettuce acquired at a hypermarket, in May of 2013.

Determination of the Minimum Inhibitory Concentration (MIC) of the vegetable *E. coli* isolate (INSali25), interpreted according to European Committee of Antimicrobial Susceptibility Testing (EUCAST, http://www.eucast.org/), revealed a non-wild-type

phenotype to colistin (MIC 16 mg/L). This isolate was also resistant to other antibiotic classes, such as penicillins, quinolones, aminoglycosides and phenicols, consistent with a multidrug resistant phenotype. Bioinformatics analyses of the *E. coli* INSali25 genome, carried out using CLC genomics workbench version 8.5.1 (QIAGEN, Aarhus) and Basic Local Alignment Search Tool (BLAST), showed the presence of additional antibiotic resistance genes, such as *aph(4)-Ia*, *estX-12, sat2, strA, strB*, *sul2*, *tetA, bla*$_{\text{TEM-1}}$, *dfrA1*, *sat2*, *floR* and *aadA1*. Characterization by Multilocus Sequence Typing (MLST), through and identification of internal fragments of *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA* genes assigned the isolate to ST1716.

Further bioinformatics analysis directed to the flanking regions of the *mcr-1* gene reinforced the significance of this finding. A 2.6Kb *mcr-1*-containing fragment on the 36,262 bp region where the gene was detected (contig_106) matched different *mcr-1*-encoding regions, such as in *Salmonella enterica* Sal25 (NZ_LFCC01000022), isolated from a food sample (Tse and Yuen, 2016) (Figure 9.1), *E. coli* 22593 (LMBK01000308) recovered from diarrheic cattle, and *S*. Typhimurium 2013LSAL04524 (LKJD01000010) collected from chicken facilities. The remaining portions of the contig where the gene was enclosed in *E. coli* INSali25 (LSRK00000106) had no resemblance with other *mcr-1* genetic environments previously described: while the upstream region of the gene harboured a partial transposon Tn*3*, including the antibiotic resistance gene *bla*$_{\text{TEM-1}}$, the downstream region of the gene showed >99% identity with contig_23 of a *S. enterica* (Tse and Yuen, 2016). In the latter region, multiple open reading frames were accommodated, among which we highlight the presence of genes encoding conjugative transfer proteins (Figure 9.1).

Globally, the variability and content of the genetic environment of the few *mcr-1* genes detected to date, suggests a high ability of the genes to mobilize and spread.

Despite previous reports on food products from animal origin, lettuce is a vegetable that is commonly consumed fresh and not subjected to any cooking process, which severely amplifies the human food safety risks involved. Moreover, the occurrence of plasmid-mediated colistin resistance in a sample that was not imported and that was acquired in a large retail store indicates the widespread distribution of *mcr-1*. Thus, we strongly suggest ending the use of colistin in agricultural settings. Our findings reinforce that mobile colistin resistance is, definitely, no longer only an Asian problem, as suggested before (Liu et al., 2016), and highlight the need to wage on a global and concerted strategy to rapidly contain the spread of this life threatening resistance mechanism.

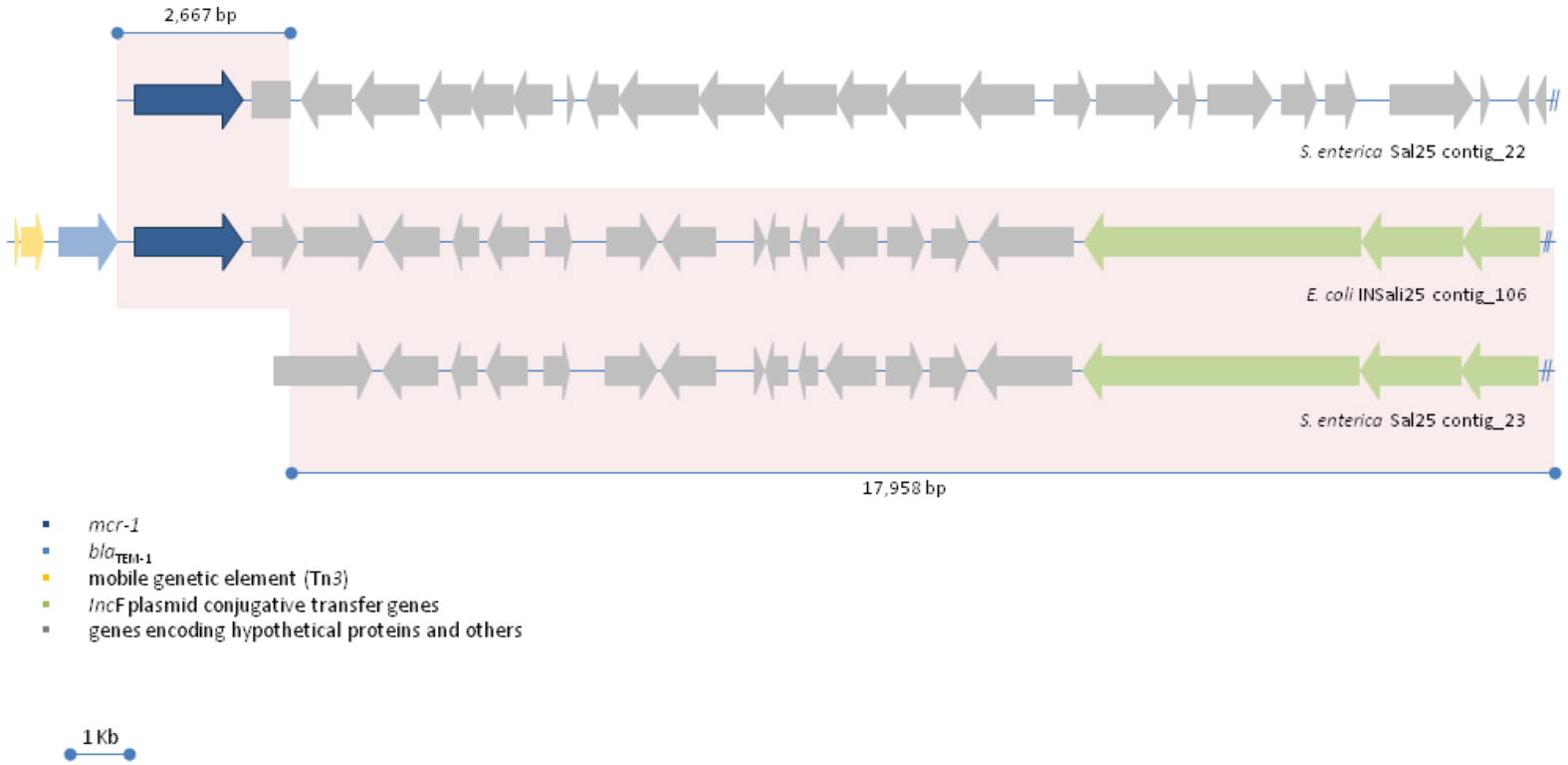

Figure 9.1. Comparison of genomic sequences harboring *mcr-1*. The CLC genomics workbench 8.5.1 (Qiagen) and the Basic Local Alignment Search Tool (BLAST) were used to compare the similarity of sequences harboring *mcr-1* gene in the two Portuguese isolates (NZ_LFCC01000000 and LSRK00000000).

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Chapter 10.

Genomic analysis of an avian multidrug resistant qnrD1-harboring Morganella morganii

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Contributions of the authors for the following manuscript:

Daniela Jones-Dias: conception and design of study, acquisition of laboratory data, analysis of data, drafting of article and critical revision of the manuscript, final approval of manuscript;
Lurdes Clemente: acquisition of laboratory and epidemiological data, critical revision of the manuscript, final approval of manuscript;
Inês Barata Moura: analysis of data, critical revision of the manuscript, final approval of manuscript;
Daniel Ataíde Sampaio: acquisition of laboratory data, final approval of manuscript;
Luís Vieira: acquisition of laboratory data, critical revision of the manuscript, final approval of manuscript;
Vera Manageiro: analysis of data, drafting of article and critical revision of the manuscript, final approval of manuscript;
Manuela Caniça: conception and design of study, drafting of article and critical revision of the manuscript, final approval of manuscript.

10.1. Abstract

Here, we report the draft genome sequence of a *Morganella morganii*, isolated in association with an *Escherichia coli*, from broilers that showed macroscopic lesions consistent with coliseptisemia. The analysis of the genome of the *M. morganii* isolate matched its multidrug resistance phenotype and enabled the identification of mobile antibiotic resistance genes, including a new plasmid carrying the plasmid-mediated quinolone resistance determinant *qnrD1*. Antibiotic resistance genes, mobile genetic elements, prophages and pathogenicity factors were also detected, suggesting the possibility of dissemination of infection in poultry industrial units. The characterization of this genome constitutes a valuable resource for the study of animal and human infections.

10.2. Introduction

The Gram negative *M. morganii* belongs to the tribe *Proteeae* of the family *Enterobacteriaceae* (O'Hara et al., 2000). This species, along with other elements of *Proteus* and *Providencia* genera can be found in the normal flora of humans, reptiles and in the wider environment (O'Hara et al., 2000; Lee and Liu, 2006; Dipineto et al., 2014). However, *M. morganii* isolates constitute clinically relevant opportunistic pathogens, which can cause an extensive variety of infections. Nosocomial outbreaks have been reported, suggesting that infections caused by *M. morganii* can lead to major clinical problems, such as wound, urinary tract infections and septicemia (Nicolle, 2001; Tsanaktsidis et al., 2003; Falagas et al., 2006; Lee and Liu, 2006; Lin et al., 2015).

This bacteria has also been associated with infections in animals, such as mammals, reptiles, and with a fatal outbreak in chickens, which suggests that *M. morganii* may also play a role as a cause of zoonotic infectious diseases (Ono et al., 2001; Choi et al., 2002; Zhao et al., 2012; Di Ianni et al., 2015).

Several factors can affect the progression and severity of an infection. In fact, the presence of pathogenicity determinants is essential to the success of *M. morganii* in any environment, particularly in animal industrial units, where the pressure caused by antibiotic treatments and the lack of prophylactic measures to avoid the spread of infectious diseases may assist (Chen et al., 2012; Lin et al., 2015). It is globally accepted that horizontal gene transfer plays an important role in the dissemination of antibiotic resistance genes and other pathogenicity-related genes (Warnes et al., 2012). Considering that *M. morganii* may share the habitat with other clinically relevant pathogens the investigation of any multidrug resistant isolate recovered from poultry is an important assignment.

The aim of this study was to investigate the molecular background sustaining the multidrug resistance and pathogenicity of a *M. morganii* isolate. Globally, we report the antibiotic susceptibility and the draft genome sequence of an avian isolate and a new *qnrD1*-harboring plasmid. The data gathered from its bioinformatic analysis might improve our understanding towards this opportunistic pathogen.

10.3. Materials and methods

Bacterial isolation, antibiotic susceptibility and molecular characterization

M. morganii INSRALV892a was recovered in association with an *E. coli* INSRALV892b isolate from a 13-days old broiler belonging to a poultry industrial unit. Samples consisted of organs collected during post-mortem examination and submitted for bacteriological analysis. During post-mortem examination, the birds showed macroscopic lesions consistent with coliseptisemia: aerosaculitis, acute enteritis, perihepatitis and fibrinous peritonitis. Suspected *Enterobactericeae* colonies obtained in MacConkey agar plates were confirmed by means of API 20E strips (bioMérieux, Marcy-l'Étoile, France).

Minimum inhibitory concentrations (MICs) were determined for both isolates by agar dilution against a panel of ten antibiotics: ampicillin, cefotaxime, ceftazidime, meropenem, ciprofloxacin, gentamicin, chloramphenicol, trimethoprim, colistin and tigecycline. To assess nonsusceptible strains, interpretation of results was performed according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org).

Plasmid mediated quinolone resistance (PMQR)-, β-lactamase-, and integrase-encoding genes were detected and identified by PCR and sequencing in both isolates, as previously described (Clemente et al., 2013).

Transferability of antibiotic resistance genes from *M. morganii* INSRALV892a and *E. coli* INSRALV892b was performed by broth mating out assays using recipient strain *E. coli* J53 $NaN3^R$. Resistant J53 *E. coli* transconjugants were then selected on MacConkey agar plates containing amoxicillin (100 mg/l) or ciprofloxacin (0.06 mg/l) together with sodium azide (200 mg/l), according with the antibiotic susceptibility profile of the donor strain. To confirm that the transconjugants acquired the antibiotic resistance genes, we detected and identified the determinants as described previously in this section.

Genomic DNA Preparation

Genomic DNA of *M. morganii* INSRALV892a was extracted using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), and DNA quantification was performed by Qubit Fluorometric Quantitation (Thermo Fisher Scientific, Waltham, MA), according with the manufacturer's instructions.

Whole-Genome Sequencing (WGS)

Libraries were prepared from 1 ng of genomic DNA using the Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA), according to the manufacturer's instructions. WGS was performed using 150 bp paired-end reads on a MiSeq (Illumina, San Diego, CA).

Genome Assembly and Annotation

Sequence reads were trimmed and filtered according to quality criteria, and assembled *de novo* using CLC genomics workbench version 8.0 (QIAGEN, Aarhus). The NCBI prokaryotic genome automatic annotation pipeline (PGAAP) was used for annotation (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/). All *de novo* contigs were BLAST searched against the GenBank's non-redundant nucleotide collection (nr/nt). PathogenFinder 1.1, ResFinder 2.1, and PlasmidFinder 1.3 were used to estimate the number of pathogenicity determinants, antibiotic resistance genes and plasmids, respectively, within the genome (Zankari et al., 2012; Cosentino et al., 2013; Carattoli et al., 2014). PHAST search web tool was used to identify and annotate any prophage sequences present in the draft genome sequence (Zhou et al., 2011). ISsaga semi-automatic annotation system was also applied to detect the presence of insertion sequences (IS) (Varani et al., 2011). RAST annotation server version 2.0 and SnapGene 3.0.3 were used for the design and annotation of the *qnrD1*-harboring plasmid (Aziz et al., 2012; Overbeek et al., 2014).

10.4. Results and discussion

M. morganii INSRALV892a was found to be nonsusceptible to ampicillin (>64mg/L), cefotaxime (>4mg/L), ceftazidime (2mg/L), ciprofloxacin (>8mg/L), chloramphenicol (16mg/L), gentamicin (>32mg/L), trimethoprim (>32mg/L), colistin (>16 mg/L) and tigecycline (0.5 mg/L), which is consistent with the phenotype of multidrug resistance. However, it should be noted that *M. morganii* is intrinsically resistant to colistin, while

tigecycline has also been shown to have poor activity against this species (http://www.eucast.org). Among the antibiotics tested, the isolate was susceptible only to meropenem (0.125mg/L). The avian *E. coli* INSRALV892b was also characterized with regard to antibiotic susceptibility and found to be nonsusceptible to ampicillin (>64mg/L), cefotaxime (>4mg/L), ceftazidime (2mg/L) and trimethoprim (>32mg/L), and susceptible to meropenem (≤0.03mg/L), ciprofloxacin (0.125mg/L), chloramphenicol (≤8mg/L), gentamicin (≤0.5mg/L), colistin (≤1 mg/L) and tigecycline (0.5mg/L). The molecular characterization of the isolates showed the presence of *qnrD1* in *M. morganii* INSRALV892a, and $bla_{\text{CTX-M-1}}$ gene flanked by an IS*Ecp1* and *orf477*, as well as a class 1 integron in *E. coli* INSRALV892b. Conjugation experiments revealed only the transference ability of $bla_{\text{CTX-M-1}}$ from *E. coli* INSRALV892b to the isogenic J53 *E. coli* strain.

The WGS assembly of *M. morganii* INSRALV892a yielded 74 contigs (each >200 bp long and >100-fold coverage), which together comprised 4,267,817bp, showing a GC content of 50.6%. The largest contig was 523,676bp long and the N50 statistic, which stands for the minimum contig length of at least 50% of the contigs, was 342,352bp. The average length of the obtained contigs was 34,190bp. Among the obtained data, six contigs, ranging from 802 and 8,575 in length and showing a minimum coverage of 117.7 fold, matched plasmid sequences of different species. Overall, the genome sequence comprised 4,116 putative genes, among which 3,950 consisted of protein encoding sequences.

In silico analysis of the antibiotic resistance genes (90% identity and 40% minimum length) revealed the presence of loci for acquired resistance to aminoglycosides (*aadA1y*, *aph(3')-Ic*, and *strA-strB*), β-lactam ($bla_{\text{OXA-1}}$), fluoroquinolones (*qnrD1*, *aac(6')-Ib-cr*), phenicols (*catA2 and catB3*), rifampicin (*arr-2*) sulphonamides (*sul2*), trimethoprim (*dfrA1*), tetracycline (*tetY)*, and streptothricin (*sat2*). The *dfrA1*, *catB2*, *sat2*, and *aadA1y* were enclosed in an In*2-17* class 2 integron that has already been described in *E. coli* from poultry (Figure 10.1A). Genes encoding resistance to tetracycline (*tetY*) and streptomycin (*strA-strB*) were detected in association with each other, and with proteins linked to DNA transfer processes; this contig's best match included sequences from *Aeromonas bestiarum* from trout and *Acinetobacter baumannii* from unknown origin (Figure 10.1B). The sulphonamide resistance gene *sul2* was enclosed in the upstream region of an 113,102bp length contig; the gene was flanked upstream by a *glmM*-containing region previously associated with a plasmid from *M. morganii*, while the downstream region consisted of a typically chromosomal region of the same bacterial species (Figure 10.1C); the *glmM* gene (formerly called *ureC*) encodes a phosphoglucosamide mutase that is considered a housekeeping gene essential for the cell wall synthesis (Tavares et al., 2003).

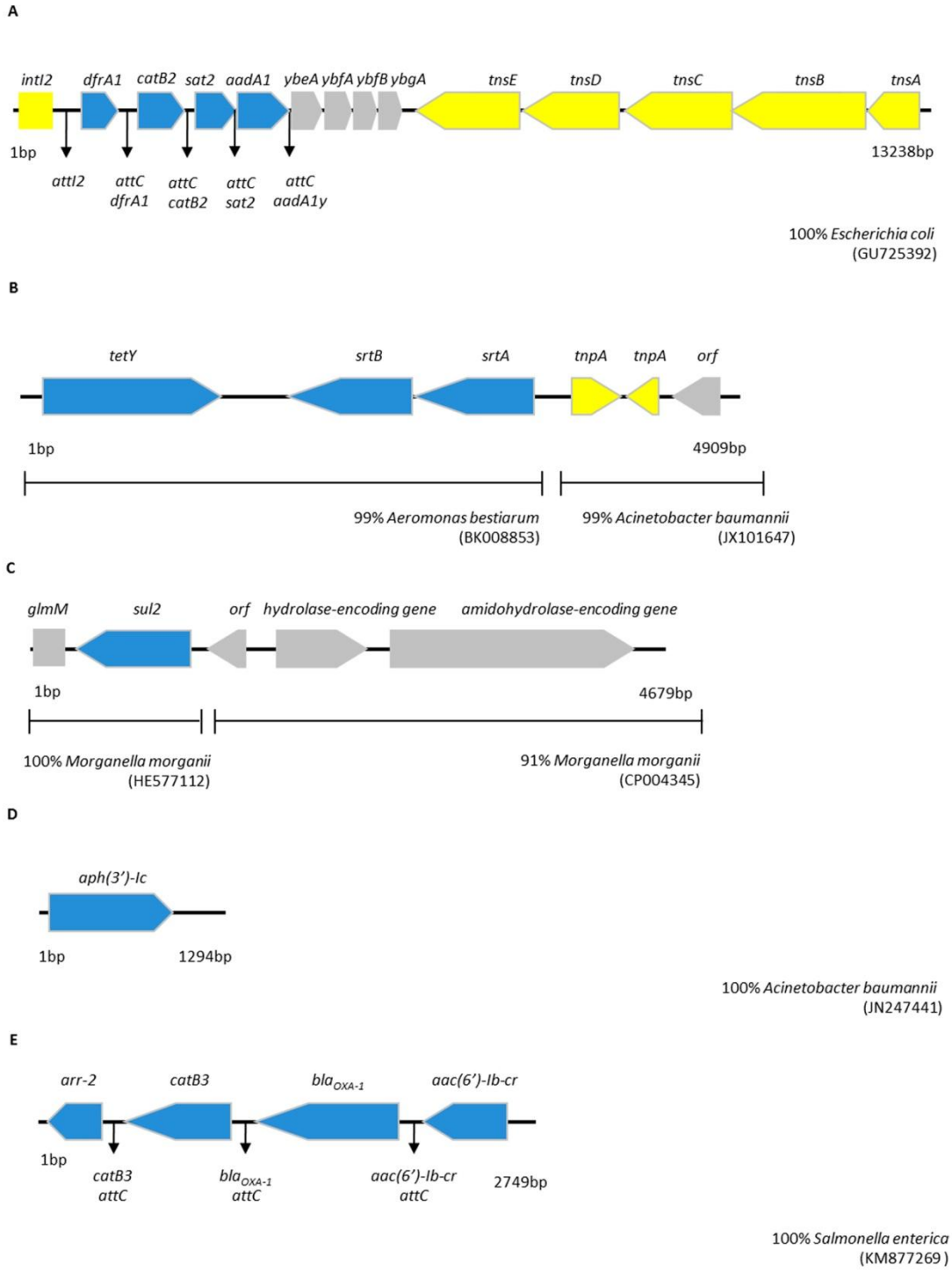

Figure 10.1. Examples of contigs containing antibiotic resistance genes in *M. morganii* INSRALV892a. (A) selection of contig 9 shows a complete In*2-17* class 2 integron encoding *dfrA1*, *catB2*, *sat2*, and *aadA1y* ; (B) selection of contig 43 encodes *tetY* and *strA-strB*, in addition to mobile genetic element and hypothetical proteins; (C) selection of contig 20 encodes *sul2* flanked by *M. morganii* genes; (D) Contig 61 consists of a small sequence accommodating *aph(3')-Ic*; (D) Contig 69 displays an integron variable region encoding *arr-2*, *catB3*, bla_{OXA-1} and *aac(6')-Ib-cr*. Blue, antibiotic resistance genes; Yellow, mobile genetic elements; Grey, other genes.

An aminoglycoside resistance gene *aph(3')-Ic* stood alone in a small 1,294bp contigs (Figure 10.1D), and the genes *arr-2*, *catB3*, bla_{OXA-1} and *aac(6´)-Ib-cr* were enclosed together, as gene cassettes of an integron variable region that has been previously found, for instance, in *S. enterica* from livestock (Figure 10.1E). Moreover, the chromosomal AmpC-encoding gene was also detected within the analyzed contigs guaranteeing resistance to β-lactam antibiotics. The genetic regions where the antibiotic resistance genes were incorporated were highly similar to other plasmid-encoded structures, previously described in different Gram negative bacteria, suggesting acquisition of resistance determinants through horizontal gene transfer (Figure 10.1).

The *qnrD1* gene was enclosed in a plasmid (pLV892) that matched an 8,449bp length contig, displaying a mean coverage of 183.9-fold and a total read count of 13,382. This mobile genetic element accommodated a 2,683bp region showing 99% homology with the previously described pCGS49 plasmid from *Proteus mirabilis* that also encodes *qnrD1* gene and three additional hypothetical proteins (Figure 10.2) (Zhang et al., 2013). Bioinformatics analyzes of the non matching region showed a gap between the two sequences corresponding to the left and right inverted repeats of IS*26*, which suggests the occurrence of a recent genetic recombination event. In addition to this difference, the plasmid detected in this *M. morganii* counted with three additional open reading frames; besides an ABC transporter-encoding gene perfectly matching a protein from *Aeromonas hydrophila*, the plasmid harboured a Tn*3* family resolvase- and transposase-encoding genes, which displayed *Shewanella baltica* as its best blast hit. According with PlasmidFinder, this new *qnrD1*-harboring plasmid pLV892 was assigned to incompatibility group Col3M. QnrD1-encoding gene is frequently located on small nonconjugative plasmids harboured by *Proteeae*, which was corroborated by our conjugation assay (Zhang et al., 2013).

Mobile genetic elements are crucial tools for the acquisition of genetic diversity, which encouraged their characterization (Warnes et al., 2012). In the *M. morganii* genome, we identified 11 prophage regions, among which six were incomplete and four were intact, comprising 381 prophage-related genes. Intact prophage regions reached between 24.2Kb and 41.7Kb and harboured 13 to 56 coding DNA sequences. The intact phages showing highest scores were assigned to Enterobacteria phage SfV, which is associated with O-antigen modification and serotype conversion in *Shigella flexneri* and Enterobacteria phage mEp235 that consists of an unclassified Lambda-like virus (Sun et al., 2013). The bioinformatic detection of IS resulted in the identification of 11 different types, which were distributed as follows: 33.3% of IS*3*, 16.7% of Tn*3* and IS*L3* and 8.3% of IS*256*, IS*6*, IS*91*, and IS*As1* (Figure S10.1). Several members of IS*3* family, which was the most

prevalent IS in the *M. morganii* genome, have been shown to be part of compound transposons. These include, for instance IS*4521*, which flanks a heat stable enterotoxin gene in enterotoxinogenic *E. coli* (Shepard et al., 2012). Besides the already mentioned *qnrD1*-harboring Col3M, no other typable plasmids were detected within the *M. morganii* genome, according with the PlasmidFinder tool.

The pathogenicity-related genes identified in the *M. morganii* genome encoded antibiotic resistance determinants and factors that influence virulence, such as mobile genetic elements and transcriptional regulators of genes involved in the metabolism, quorum sensing and motility; 22 different pathogenic families were included that showed a 68.9% certainty of the isolate being a human pathogen.

Multidrug resistant *M. morganii* isolates are rare and normally associated with non invasive human nosocomial opportunistic infections (Nicolle, 2001; Falagas et al., 2006). The detection of an avian *M. morganii* isolate harboring multiple and mobile antibiotic resistance genes and pathogenicity factors raises concerns regarding the dissemination of infection in birds and potential risk of zoonotic transmission. Moreover, colonization and/or infection of the same host by more than one bacterium have previously been associated with *in vivo* transference of genetic material between bacteria (Manageiro et al., 2014; Göttig et al., 2015), which may justify the homology between sequences from different species. Several factors may affect the susceptibility of poultry to bacterial diseases, being environmental stressors and previous antibiotic treatments crucial to the development of infections with many *Enterobacteriaceae* (Burkholder et al., 2008).

10.5. Conclusion

There is a growing interest in antibiotic resistance from non-human sources and its impacts on human health and in the environment (Cantas et al., 2013). *M. morganii* is a well characterized opportunistic pathogen (Lee and Liu, 2006). However, their detection in poultry flocks, co-habiting the same hosts as other clinically important pathogens, makes it susceptible for the acquisition of pathogenicity factors by horizontal gene transfer (Warnes et al., 2012). Moreover, the detection a new *qnrD1*-harboring plasmid that incorporates sequences from similar resistance plasmids and DNA from other environmental sources suggests the occurrence of recent recombination events.

To the best of our knowledge this report represents the first genome analysis of an isolate from animal origin carrying QnrD1. The genome sequence represents a valuable resource for studies on the epidemiology of zoonotic *M. morganii* isolates, and will serve as a tracker of the dissemination of antibiotic resistance.

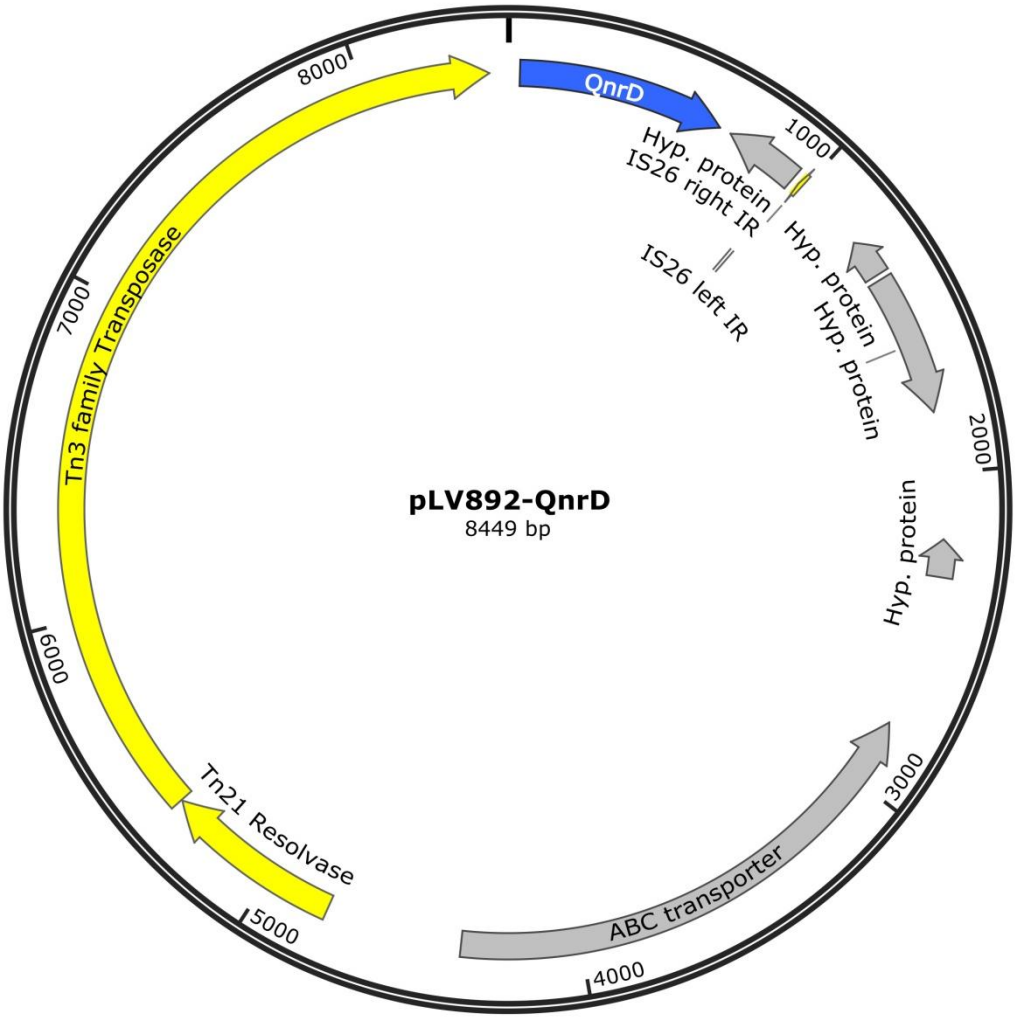

Figure 10.2. *qnrD1*-harboring plasmid pLV892 from *M. morganii*. Hyp, Hypothetical. Blue, antibiotic resistance genes; Yellow, mobile genetic elements; Grey, other genes.

10.6. Data access

This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession LGYC00000000. The version described in this paper is version LGYC01000000.

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Chapter 11.

Draft genome sequence of a pathogenic O86:H25 sequence type 57 Escherichia coli strain isolated from poultry and carrying 12 acquired antibiotic resistance genes

This research paper was published as:

***Daniela Jones-Dias**, Vera Manageiro, Daniel Ataíde Sampaio, Luís Vieira, Manuela Caniça. 2015. Draft genome sequence of a pathogenic O86:H25 sequence type 57 Escherichia coli strain isolated from poultry and carrying 12 acquired antibiotic resistance genes. **Genome Announcements.** 3: e01107-15.*

Contributions of the authors for the following manuscript:

Daniela Jones-Dias: conception and design of study, acquisition of laboratory data, analysis of data, drafting of article and critical revision of the manuscript, final approval of manuscript;
Vera Manageiro: analysis of data, critical revision of the manuscript, final approval of manuscript;
Daniel Ataíde Sampaio: acquisition of laboratory data, final approval of manuscript;
Luís Vieira: acquisition of laboratory data, critical revision of the manuscript, final approval of manuscript;
Manuela Caniça: conception and design of study, critical revision of the manuscript, final approval of manuscript.

11.1. Abstract

Escherichia coli is a commensal bacteria that is frequently associated with multidrug resistant zoonotic and foodborne infections. Here, we report the 5.6Mbp draft genome sequence of an *E. coli* recovered from poultry, encoding multiple acquired antibiotic resistance determinants, virulence factors, pathogenicity determinants and mobile genetic elements.

11.2. Main text

E. coli is the most prevalent commensal microorganism of the human and animal gastrointestinal tract, remaining one of the most frequent causes of bacterial infections worldwide (Allocati et al., 2013). Diseases caused by this pathogen are difficult to treat due to the presence of antibiotic resistance determinants that regularly render the isolates multidrug resistant (Ikram et al., 2015). Moreover, antibiotic resistance genes are frequently inserted in mobile elements that facilitate its transmission between bacteria (Feng et al., 2015; Göttig et al., 2015).

E. coli INSLA289 was isolated from broilers belonging to an animal farming facility with unknown clinical history, and was recovered from macerates of organs during post-mortem examination. It was tested for its antibiotic resistance and found to be nonsusceptible to penicillins, first, second, third, and fourth generation cephalosporins, aztreonam, quinolones, tetracycline, aminoglicosydes and nitrofurantoin. Genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Aarhus). Libraries were prepared from 1ng of genomic DNA using the Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA) according to the manufacturer's instructions. WGS was performed using 150 bp paired-end reads on a MiSeq (Illumina, San Diego, CA). Sequence reads were then trimmed and filtered according to quality criteria, and assembled *de novo* using CLC genomics workbench version 8.0 (QIAGEN, Aarhus). The NCBI prokaryotic genome automatic annotation pipeline (PGAAP) was used for annotation. PathogenFinder 1.1, ResFinder 2.1, VirulenceFinder 1.4, SerotypeFinder 1.1 and MLST 1.8 were used to estimate pathogenicity determinants, antibiotic resistance genes, virulence factors, MLST and serotype of this isolate, respectively (Larsen et al., 2012; Zankari et al., 2012; Cosentino et al., 2013; Joensen et al., 2014).

The draft genome of the *E. coli* INSLA289 was assembled *de novo* into 300 contigs (each >200 bp long), which together comprised 5,584,816bp. Global results indicated a GC content of 50.6%, an average coverage of 175.2 and a N50 of 99,169bp. The largest

obtained contig was 444,397bp, presenting a coverage of 131.7-fold. The obtained contigs were searched against the GenBank database nucleotide collection (nr/nt) using Megablast. Globally, a total of seventy contigs matched multiple plasmid sequences therein deposited.

ResFinder 2.1 (90% identity and 40% minimum length) enable the detection of 12 antibiotic resistance genes: $bla_{\text{CTX-M-1}}$ (contig 77), $bla_{\text{SHV-12}}$ (contig 179), $bla_{\text{TEM-116}}$ (contig 193), *aadA1y* (contig 20), *aph(3')-Ic* (contig 172), *strA* and *strB* (contig 172), *sul2* (contig 72), *dfrA1* (contig 20), *tetA* (contig 188), *tetB* (contig 99), and *sat2* (contig 20). This ST57 *E. coli* isolate also carried an IS*10*-disrupted In*2-4* class 2 integron, where *dfrA1*, *sat2*, and *aadA1y* were accommodated, resulting in the disruption of *attI2* integration site. Seven virulence factors were also detected: *cma* (contig 154), *ireA* (contig 98), *prfB* (contig 9), *ioN* (contig 89), *tsh* (contig 86), *iss* (contig 89), and *iha* (contig 20). Moreover, the isolate expressed serotype O86:H25 and displayed a prediction of 91.4% for being a human pathogen.

This draft genome sequence constitutes a valuable resource for international genomic comparison studies and may be helpful to identify genomic traits associated with the zoonotic potential of multidrug resistant *E. coli* isolates.

11.3. Data access

This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession LHAT00000000. The version described in this paper is version LHAT01000000.

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Chapter 12.

Quantitative proteome analysis of an antibiotic resistant Escherichia coli exposed to tetracycline reveals multiple affected metabolic and peptidoglycan processes

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Rune Matthiesen: conception and design of study, analysis of data, critical revision of the manuscript, final approval of manuscript.

** these authors contributed equally to this work.*

12.1. Abstract

Tetracyclines are among the most commonly used antibiotics administrated to farm animals for disease treatment and prevention, contributing to the worldwide increase in antibiotic resistance in animal and human pathogens. Although tetracycline mechanisms of resistance are well known the role of metabolism in bacterial response to antibiotic stress could contribute to the development of novel targets for multidrug resistant microorganisms. In this study spectral counts-based label free quantitative proteomics has been applied to study the response to tetracycline of the environmental-borne *Escherichia coli* EcAmb278 isolate soluble proteome. A total of 1,484 proteins were identified by high resolution mass spectrometry at a false discovery rate threshold of 1% of which 108 were uniquely identified under absence of tetracycline, whereas 126 were uniquely identified in presence of tetracycline. These proteins revealed interesting difference in e.g. proteins involved in peptidoglycan based cell wall proteins and energy metabolism. Upon treatment, 12 proteins were differentially regulated showing more than 2-fold change and p <0.05 (p value corrected for multiple testing). This integrated study using high resolution mass spectrometry based label-free quantitative proteomics to study tetracycline antibiotic response in the soluble proteome of resistant *E. coli* provides novel insight into tetracycline resistance.

12.2. Introduction

Infections due to antibiotic resistant pathogens constitute a major public health concern, frequently leading to high levels of morbidity, mortality and healthcare costs (Giske et al., 2008; Laxminarayan et al., 2013).

Tetracyclines are a group of broad-spectrum antibiotic agents that exhibit antibiotic activity against a wide range of microorganisms. Their cost, favorable antimicrobial properties, and absence of major adverse side effects have led to their extensive use in human and veterinary medicine, as well as in agriculture (Roberts, 2003). Tetracyclines sterically block aminoacyl-tRNA binding within the bacterial ribosome, inhibiting protein synthesis. However, this association of the antibiotics with the ribosomes is reversible, hence explaining their bacteriostatic effect (Chopra and Roberts, 2001). Resistance to tetracyclines may be transfered or arise by chromossomal mutations, and occurs through five main mechanisms: 1) production of ribosomal protection proteins (RPPs), 2) active efflux of tetracycline from the cell, 3) enzymatic inactivation of the antibiotic, 4) decreased drug permeability 5) and mutation of the antibiotic target (Vranakis et al., 2014). There

are currently over 40 different acquired tetracycline resistance determinants recognized: 38 *tet* (tetracycline resistance) genes and 3 *otr* (oxytetracycline resistance) genes (Roberts, 2003).

Considering the existing resistance to tetracycline, new generation tetracycline-based antibiotics are now starting to be developed and showing potential for the treatment of serious multidrug resistant Gram negative infections, which highlights the importance of preserving this class of antibiotics (Mawabo et al., 2015; Trudy et al., 2015). Moreover, it has been reported that tetracyclines induce the expression of multidrug resistance efflux pumps, ribosomal proteins, and iron uptake transporters, which may favor the emergence of resistance to other antibiotic classes (Yun et al., 2011).

Additionally, there are emerging concerns on the ability of human actions to enhance the selection pressure caused by antibiotics, which may increase the mobility of antibiotic resistance genes, and their recruitment by clinically relevant pathogens (Finley et al., 2013). Previous studies have already been able to establish that agriculture soils may become contaminated with clinically important antibiotics, antibiotic resistant isolates and antibiotic resistance genes through the application of manure originated from antibiotic treated livestock (Hartmann et al., 2012). Moreover, specific practices, such as intensive agriculture, are known to provide major impacts on the selection of environmental-borne resistant genes, as they may supply a selective pressure, either by direct application of antibiotics on crops (such as tetracycline, flumequine or streptomycin) or by indirect exposure through manure or wastewater amendments (Popowska et al., 2012). Thus, the combination of soil contamination with the use of tetracyclines in agriculture may be creating the ideal conditions to favor the selection and emergence of clinically important mobile and non-mobile antibiotic resistant mechanisms.

The role of metabolism in the bacterial response to antibiotics has recently gained interest due to the lack of novel targets for multidrug resistant microorganisms. Many studies have already focused on the evaluation of variations in protein expression caused by antibiotic exposure in tetracycline resistant microorganisms from different species (Xu et al., 2006; Yun et al., 2011; Vranakis et al., 2012). Furthermore, most of the previous proteomics studies have targeted the membrane proteome and to date there are no quantitative tetracycline resistance studies performed with the latest generation of high resolution mass spectrometers allowing high mass accuracy in both Mass Spectrometry (MS) and MS/MS scans. Additionally, little is known about the metabolic response of genetically resistant bacterial populations to antibiotic exposure (Bhargava and Collins, 2015).

The changes that occur within antibiotic resistance soil bacteria, when challenged with a stressor such as an antibiotic, are reflected in their proteome, which can change in

response to any cellular or external environmental factor (Burchmore, 2014; Vranakis et al., 2014). Thus, the use of proteomic profiling to identify molecules that might be directly or indirectly related with the response to antibiotic exposure represents an important step in determining the metabolic pathways that might be associated with antimicrobial activity. By highlighting pathways involved in the acquisition of resistance, and which may themselves represent resistance-proof drug targets, these approaches may be helpful not only to extend the usefulness of current antimicrobials but also to develop new drugs. Moreover, the mechanisms that enable bacteria to survive antibiotic exposure long enough to resist, might themselves present resistance-proof drug targets (Burchmore, 2014).

In this study, we used a liquid chromatography-mass spectrometry-based (LC-MS/MS) proteomics approach to evaluate the global metabolic changes in the soluble protein fraction of an antibiotic resistant *E. coli* isolate, when challenged with tetracycline. The MS analysis was performed using high mass resolution in MS and MS/MS scans and label free quantitation using three biological replicas for both control and treated cells.

12.3. Materials and methods

Characterization of bacterial isolate

Isolate *E. coli* EcAamb278 was collected in July of 2012, from a soil sample aseptically recovered from an agricultural setting near Almeirim, Portugal, used for the intensive farming of tomato plants, as previously described (Jones-Dias et al., 2016).

The nonsusceptibility profile of the isolate, performed according to the Antibiogram Committee of the French Society of Microbiology (SFM) (Bonnet et al., 2013), comprised penicillins, first, second, third and fourth generation cephalosporins, monobactam, β-lactam/β-lactamase inhibitors and tetracycline. Previous genomic characterization, performed as described (Jones-Dias et al., 2015b), confirmed the presence of five antibiotic resistance genes encoding the penicillin TEM-135, the extended-spectrum β-lactamase CTX-M-1, the dihydropteroate synthase Sul2 and the efflux pumps TetA and TetB. Furthermore, the *E. coli* EcAamb278 harboured four virulence factors (Gad, Iss, IroN, Cma) and displayed a 93.1% probability of being a human pathogen, according to PathogenFinder (Cosentino et al., 2013). The multilocus sequence typing (MLST) method defined the isolate as a ST1718. Serotype determination, performed upon the analysis of FliC-, Wzt- and Wzm-encoding genes, showed an *E. coli* from serotype O9:H31.

Bacteria and culture conditions

Frozen glycerol stocks of *E. coli* EcAmb278 were seeded onto MacConkey agar and trypticase soy agar plates sequentially, and grown for 18h, at 37ºC. Single colonies of the *E. coli* EcAmb278 were pre-inoculated in 5ml of Brain Heart infusion broth that was then used to inoculate 250ml of the same medium, in covered 500ml Erlenmeyer flasks at 37 °C, with rotary aeration at 180rpm. For three out of the six experiments, tetracycline (Sigma-Aldrich) was added to the culture in a final concentration of 10 mg/L, while the three remaining replicates were cultured without exposure to antibiotics. The cells were then harvested during the exponential phase (OD640nm= 0.6, approximately 2×10^9 cells/ml).

Extraction of soluble proteins and protein quantification

Proteins were isolated using a protein extraction method for soluble proteins. Briefly, the bacterial cells were harvested at 10,000 g for 5 min at 4 °C. Three biological replicates of three independent assays were performed with and without antibiotic. The pellet was then completely resuspended in MES-NaOH 20mM pH 8.0 and centrifuged three times using the conditions above mentioned. After centrifugation, bacterial pellets were resuspended in 10ml for each 2.5g of cells in MES-NaOH 20mM pH 8.0, DTT 1mM, with addition of bacterial protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), and disrupted by ultrasonic treatment as previously described (Mendonça et al., 2008) and stored at −20 °C until further analyses. Protein concentration was estimated using BCA protein assay kit (Pierce, Rockford, IL), according to the manufacturer's instructions.

One-dimensional electrophoresis

The integrity and reproducibility of the bacterial proteome was estimated by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as previously described (Mendonça et al., 2008). Briefly, approximately 15µg of protein extract was collected and resuspended in an equal volume of sample buffer containing 0.5MTris-HCl 1M pH 6.8, glycerol, SDS, DTT and bromophenol blue.

One-dimensional gel electrophoresis was then conducted on vertical SDS-polyacrylamide gels with final acrylamide concentrations of 12 and 5% (wt/vol) for the separating and the stacking gels, respectively, in an ENDURO VE20 Vertical Gel Electrophoresis System (Labnet International, Edison, NJ), as previously described (Laemmli, 1970). Proteins were separated with a constant current of 125V until the dye-front reached the bottom of the gel and stained with Coomassie Blue R-250 (Sigma-Aldrich, St. Louis, MO).

Peptide Sample Preparation

Protein solution containing SDS and DTT were loaded into filtering columns and washed exhaustively with 8M urea in HEPES buffer. Proteins were then incubated overnight with trypsin sequencing grade (Promega, Madison, WI) after alkylation with iodoacetamide and reduction with DTT.

Mass Spectrometry

Peptides generated as described above were desalted and concentrated (Carvalho et al., 2014) prior to analysis by nano LC-MS/MS using a Q-Exactive (Thermo, San Jose, CA) mass spectrometer coupled to a Dionex NCP3200RS HPLC setup (Thermo, Sunnyvale, CA). A 75 m ID, 15 cm in length home build reversed phase column (Reprosil-pur C18-AQ 3 m, Ammerbuch-Entringen, Germany) was used to separate peptides. The analytical gradient was generated at 200 nL/min increasing from 5% Buffer B (0.1% formic acid in acetonitrile)/95% Buffer A (0.1% formic acid) to 35% Buffer B/65% Buffer A over 110 min followed by an increase to 90% Buffer B/10% Buffer A during 10 min. MS survey scans were scanned from m/z 350 to m/z 1400 at 70,000 resolution (AGC: 1e6 and Maximum IT: 120 ms). An upper limit of 20 most abundant ions was subjected to MS/MS and measured at a resolution of 35,000 (AGC: 5e4 and Maximum IT: 120 ms) with lowest mass set to m/z 100.

Preprocessing of MS data

Q-Exactive data was calibrated using polycyclodi-methylsiloxane (PCMs—out gassed material from semiconductors) present in the ambient air and Bis (2-Ethylhexyl) (Phthalate) (DEHP—from plastic) (Schlosser and Volkmer-Engert, 2003; Olsen et al., 2005) modular VEMS (Matthiesen, 2013b). VEMS further allows alternative parent ion annotations for each MS/MS spectrum which is needed if two peptide elution profiles overlap in the m/z and retention time dimension. These alternative parent ion annotations were taken into account during the database dependent search.

MS database dependent search

All data were searched with VEMS (Matthiesen, 2013a; Carvalho et al., 2014). Mass accuracy was set to 5 ppm for peptides and 10 mDa for peptide fragments. Gaussian weight for fragment ions was set to 5 and the six most intense fragment ions per 100 Da was used for scoring fragment ions. Four missed cleavages were specified. The data was first searched against two databases: all bacterial protein sequences in NCBI and all

bacterial protein sequences in UniProtKB/TrEMBL (Release 2015_02). These searches confirmed that the peptide spectra assignments were mainly matching bacterial proteins from *E. coli*. The search was therefore repeated only against *E. coli* proteins in UniProtKB (Figure S12.1). The three databases included permutated protein sequences, leaving Lys and Arg in place, together with common contaminants such as human keratins and proteases (Bunkenborg et al., 2010).

Fixed modification of carbamidomethyl cysteine was included in the search parameters. A list of 5 variable modifications (Figure S12.2) was considered for all data against the full protein database. Protein N-terminal Met-loss is not specified for VEMS searches since VEMS by default checks N-terminal Met-loss. The false discovery rate (FDR) for protein identification was set to 1% for peptide and protein identifications. No restriction was applied for minimal peptide length. Identified proteins were divided into evidence groups as defined by Matthiesen *et al*. (Matthiesen et al., 2012).

Quantitative proteome analysis

Proteins were quantified by spectral counting (Matthiesen and Carvalho, 2013) and mziXIC (Matthiesen, 2013b) followed by intensity-based absolute quantification (iBAQ) (Mann and Edsinger, 2014) estimation. For statistical comparison of regulated proteins between control and tetracycline treated spectral counting data was used. No imputation for missing values was used. This leads to less significant proteins than if imputation was performed, however, this raises the confidence on the proteins identified as statistical significant. The quantitative spectral count values were added one and log two transformed. The quantitative values were next normalized using quantile normalization and statistical calculation of *p* values was performed by the R package limma (Figure S12.3). Correction for multiple testing was done by the required false discovery rate (FDR) method (Benjamini and Hochberg, 1995).

Validation of significant regulated proteins

To further validate the significant regulated proteins based on spectral counting all peptide spectra assignments using raw data were depicted together with delta mass plots and subsequently manually scrutinized (See Figure S12.4). Validation of spectral counts was made by automatically extracting the ion counts using a mass accuracy of 0.005 Da and maximum allowed deviation from expected isotope distribution of 10%. The quantitation obtained by ion counts are summarized in Figure S12.5 and confirms all the quantitative values obtained by spectral counting on the significant regulated proteins.

Functional analysis of proteins

Functional enrichment analysis was performed using DAVID Bioinformatics server (Huang da et al., 2009). All identified proteins, proteins unique for control and for tetracycline treated were submitted to DAVID using *E. coli* proteome as background. Gene ontology tables with the functional enrichment results for biological processes (BP), cellular component (CC) and molecular function (MF) were manually downloaded for the above mentioned three sets of proteins. The tables were sorted on based on FDR corrected P value. The tables were filtered by starting with the most significant enriched functional category and then eliminating any subsequent functional category with more than 80% protein overlap with any of more significant enriched functional categories. We find that 80% is good trade off to eliminate redundant functional categories. Finally, the ten most significant functional categories were depicted using barplot in the statistical programming language R (R-Core-Team, 2014). FDR corrected *p* values were used for the barplots.

Proteome Xchange Accession Numbers

The raw MS data obtained from the proteome of isolate EcAmb278 has been deposited at Proteome Xchange under the accession PDX00000.

12.4. Results and discussion

Quantitative proteome analysis of environmental-borne *E. coli* EcAmb278 in the presence and absence of tetracycline

The use of broad-spectrum tetracyclines as a growth promoter in agriculture and as an infection control agent in domestic animals, aquaculture, and horticulture have contributed, among other factors, to its widespread resistance. We have previously reported the presence of high level antibiotic resistant isolates on a collection of nonsusceptible Gram negative bacteria recovered from sites of different agricultural practices (see also section *Characterization of bacterial isolate* of *Materials and methods* section for more details on EcAmb278) (Jones-Dias et al., 2016). *E. coli* EcAmb278 isolate (Gram negative) was recovered from soils of intensive agriculture. MS-based proteomics analysis of tetracycline resistance has targeted mainly the pathogens membrane components. Several studies point to the necessity for further investigation of the role of metabolic pathways in drug resistance (Vranakis et al., 2014; Xie et al., 2015). We present

here the first in depth analysis of the soluble proteome of a nonsusceptible *E. coli* with the highest proteome coverage using high resolution mass spectrometry. Previously use of cumbersome fractionation and sample preparation methods are less relevant with the use of newly accurate and sensitive instruments allied with powerful bioinformatics tools. We have analyzed the soluble proteome of *E. coli* EcAmb278 itself and challenged with 10 mg/L tetracycline followed by MS-based label free quantitation. The MS data was acquired with high accuracy in both MS and MSMS scans.

In both conditions analyzed (in the presence and absence of tetracycline) a total number of 1,484 proteins was identified, using a 1%FDR as cut off and collapsing the proteins to encoding genes to remove bacterial strain redundancy. The UniProt annotation, quantitative and statistical result on the identified proteins is available in Table S12.1 both before and after collapsing the proteins into gene encoding proteins. The Uniprot bioinformatics database (http://www.uniprot.org/) and the ExPASy SIB Bioinformatics Resource Portal (http://www.expasy.org/), complemented with an in-depth literature search, were used to determine protein's functions and its association to specific biological processes at cellular level.

A total of 37% (547 proteins out of 1,484) proteins identified are annotated to a cellular component (Figure 12.1A). The most significantly enriched component is cytosol (GO:0005829), which reflects the targeted soluble protein fraction described in the method section. However, we do also, to a less extent, identify proteins associated to periplasmic space and membranes (Figure 12.1A). Ribonucleoprotein complex members such as the ribosomal protein family are significantly represented as bacteria do not have a distinct nucleus that separates DNA from ribosomes, so there is no barrier to immediate translation. The ribosome is an important target for a wide variety of antibiotics. Tetracycline blocks the binding of aminoacylated tRNA (aa-tRNA) to the A-site of the 30S subunit, inhibiting protein synthesis (Brodersen et al., 2000; Pioletti et al., 2001). GO categories translation (GO:0006412) and in the GO annotation categories biological process (Figure 12.1B) and molecular function structural constituent of ribosome (GO:0003735) are the primary significantly enriched for identified proteins (Figure 12.1C). Generation of precursor metabolites and energy (GO:0006091) is also significantly identified and we can consequently address the hypothesis put forward by Liu *et al* (Liu et al., 2014).

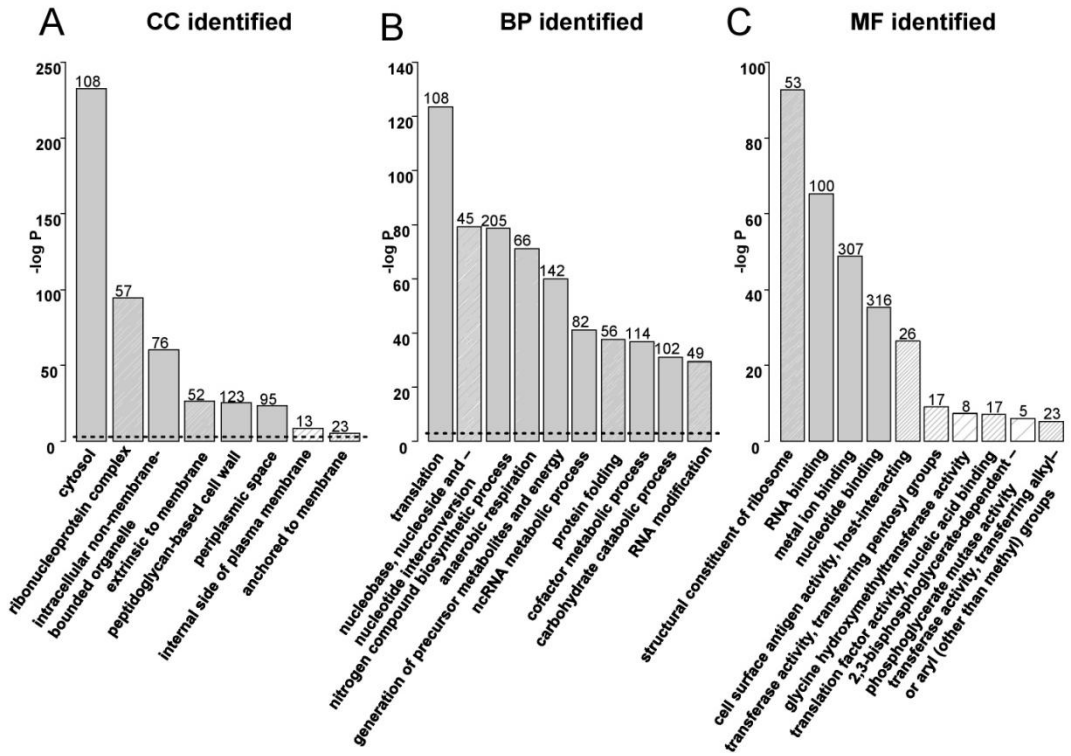

Figure 12.1. Functional enrichment analysis of all identified soluble proteins from *E. coli* EcAmb278 cultured in the presence and absence of tetracycline. (A) The proteins are analyzed according to cellular component (CC), (B) biological process (BP) and (C) molecular function (MF). The probability of a protein being enriched in a specific subcategory is represented by –log *p*, *p* corresponding to the *p* value. The values on top of each bar correspond to the number of genes enclosed in the specific subcategory which correlates with the pattern fill.

Comparison of identified proteins in controls versus tetracycline treated *E. coli* EcAmb278

The comparison of identified proteins in absence and presence of tetracycline is depicted in Figure 12.2. A total of 1,484 proteins were identified of which 108 were uniquely identified under absence of tetracycline whereas 126 were uniquely identified in presence of tetracycline.

It has been hypothesized that changes in energy metabolism could be important cell survival response upon antibiotics (Liu et al., 2014; Lobritz et al., 2015). Our results provide support for this hypothesis since we observe enrichment of e.g. “aerobic respiration”, “phosphate transport” and “ATPase activity” among proteins identified uniquely in the absence of tetracycline (Figures S12.6 and S12.7). These findings are in

accordance with previous metabolomics studies demonstrating that antibiotic growth inhibition is associated to down regulation of bacterial cellular respiration (Lobritz et al., 2015).

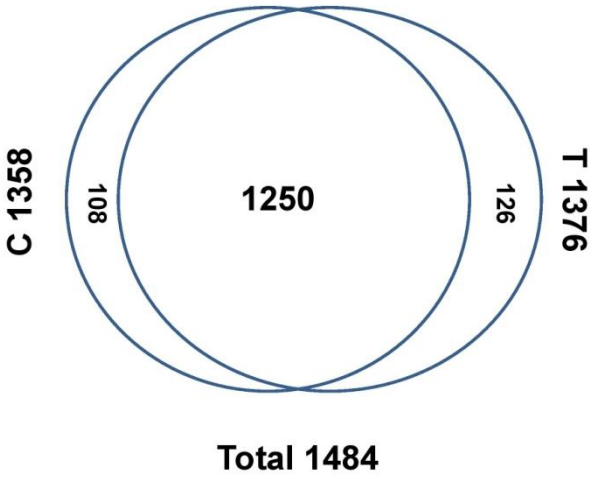

Figure 12.2. Venn diagram comparing identified proteins in control and tetracycline treated *E. coli.*

Proteins identified uniquely in presence of tetracycline (see Figure S12.7) were enriched in proteins involved in catabolic processes. We speculate that tetracycline's effect on ribosome translation cause damage that needs to be abolished by catabolic processes. Furthermore, proteins unique for *E. coli* in the presence of tetracycline were enriched in gene ontology category manganese ion binding (Figure S12.7C).

A set of peptidoglycan-based cell wall proteins were unique for control and others for tetracycline treated (Figures S12.6A and S12.7A). Altered permeability of cell wall is one of the mechanisms described to obtain tetracycline resistance and our results provided evidence that this specific alteration can potentially occur by shifting to another set of proteins involved in peptidoglycan-based cell wall rather than just upregulating additional peptidoglycan-based cell wall proteins (see table S12.1, sheet "peptidoglycan-based cell wall"). For example, the tyrosine-protein kinase (*etk*) was only identified in presence of tetracycline. Etk and Wzc are essential for synthesis of specific extracellular polysaccharide (Shi et al., 2010).

We have also explored the presence of the post-translational modification acetylation (Figure S12.2 and S12.8). Post-transcriptional and post-translational processes generate a remarkable diversity of mature proteins from a single gene that can influence the global metabolic and evolutionary responses of a cell (Baer and Millar, 2015). In this study, lysine acetylation appeared to be more frequent in the tetracycline treated EcAmb278 isolate

although not significantly different. N-terminal protein acetylation was less frequently detected, compared to control conditions.

Significantly differentially regulated *E. coli* proteins under tetracycline treatment

The comparison of the proteome profiles of the two *E. coli* EcAamb278 samples pointed to several proteins with altered expression under tetracycline stress conditions. Among those, the twelve most significant proteins (FDR-corrected $p \leq 0.05$), differentially regulated by more than two-fold between the tested conditions, were further analyzed, and are showed in Table 12.1 and Figure 12.3. The identified proteins, which were either up-regulated or down-regulated in the tetracycline exposed strain, compared to the non-exposed control, are involved in the following biological functions: DNA replication/repair (Q8XBL5 and Q8FH89), transcription (P64624 and B7UQK0), virulence (P61887 and P08191) intracellular trafficking and secretion (P20966), biosynthesis of vitamins (P0AG42), other biosynthesis processes (P0A8Z1 and Q46925), other processes (P22256), and unknown function (P0AD11) (Figure 12.3). Among those, the proteins directly or indirectly related with resistance mechanisms or virulence will be further discussed here.

The low number of significantly regulated proteins could be explained by the fact that nearly 26% of the most abundant proteins had functions related to protein or amino acid biosynthesis. In other words, we might argue that among these biological processes no regulation was needed because the existing proteins were abundant enough to accomplish the cellular needs. In fact, since little regulation was found for these protein categories we might conclude that these protein groups do not seem to be directly relevant for survival of *E. coli* EcAamb278 upon tetracycline treatment.

The acyl-CoA thioester hydrolase (P0A8Z1) is involved in biosynthesis of coenzyme A, which has been implicated in resistance to aminoglycosides, being responsible for a conformational increase in the binding site of an aminoglycoside modifying-enzyme (Hu et al., 2011). This group of antibiotics displays a mode of action that, just like tetracycline, discontinues protein biosynthesis (Davies, 2006). Moreover, a previous study focused on the quantitative proteome of *Coxiella burnettii* upon treatment with a tetracycline antibiotic, also showed an increased expression of an enzyme involved in the synthesis of coenzyme A (Vranakis et al., 2012). Thus, the fact that the acyl-CoA thioester hydrolase is here significantly up-regulated might suggest its involvement in tetracycline resistance.

Table 12.1. Proteins significantly up- and down-regulated in *E. coli* EcAmb278 challenged with 10 mg/L of tetracycline.

Accession number	Strain	Biological process	Protein	Function	Gene	Log2 ratio	FDR-corrected *p* value	Reference
Up-regulated proteins								
P0A8Z1	*E. coli* O6:H1 ATCC700928	Other biosynthesis processes	Putative acyl-CoA thioester hydrolase	Catalyzes the hydrolysis of the thioester bond in palmitoyl-CoA and malonyl-CoA Catalyzes the hydrolysis of the thioester bond in palmitoyl-CoA and malonyl-CoA	*yciA*	2,3	1,86E-61	Willis et al., 2008
Q8XBL5	*E. coli* O157:H7	DNA repair/replication	DNA ligase	Catalyzes the formation of phosphodiester linkages between 5'-phosphoryl and 3'-hydroxyl groups in double-stranded DNA; essential for DNA replication and repair of damaged DNA	*ligA*	4,7	0,000384	Mills et al., 2011
Q46925	*E. coli* O157:H7	Other biosynthesis processes	Cysteine sulfinate desulfinase	Functions as a selenium delivery protein in the pathway for the biosynthesis of selenophosphate; seems to participate in Fe/S biogenesis by recruiting the SufBCD-SufE proteins	*csdA*	3	0,002996	Trotter et al., 2009

P61887	*E. coli* K12	Virulence	Glucose-1-phosphate thymidylyltransferase	Catalyzes the formation of dTDP-glucose, from dTTP and glucose 1-phosphate, as well as its pyrophosphorolysis; involved in O-antigen biosynthesis	*rmlA*	3,4	0,00494	Erbel et al., 2003
Q8FH89	*E. coli* O6:H1 ATCC700928	DNA repair/replication	Pyridoxamine kinase	Functions in a salvage pathway using pyridoxamine and phosphorylating B6 vitamers	*pdxY*	2,5	0,017328	Yang et al., 1998
P64624	*E. coli* K12	Transcription	Predicted transcriptional regulator	Unknown function	*yheO*	3,2	0,017462	-
P0AG42	*E. coli* O157:H7	Biosynthesis of vitamins	Riboflavin biosynthesis protein	Involved in the riboflavin (vitamin B2) biosynthesis pathway as kinase and transferase	*ribF*	3,2	0,017717	Gutiérrez-Preciado et al., 2015
P0AD11	*E. coli* O6:H1 ATCC700928	Unknown	Hypothetical protein	Unknown function	*yecJ*	2,8	0,017717	-
Down-regulated proteins								

P20966	*E. coli* K12	Intracellular trafficking and secretion	PTS system fructose-specific EIIBC component	Catalyzes the phosphorylation of incoming sugar substrates concomitantly with their translocation across the cell membrane; involved in fructose transport	*fruA*	-3,4	0,002996	Postma et al., 1993
B7UQK0	*E. coli* O127:H6 E2348/69	Transcription	HTH-type transcriptional regulator	L-Ascorbate is utilized under anaerobic conditions through proteins encoded by the ula regulon, which is under the control of the*ulaR* repressor	*ulaR*	-2,8	0,00494	Garces et al., 2008
P08191	*E. coli* K12	Virulence	Bacterial adhesin	Involved in regulation of length and mediation of adhesion of type 1 fimbriae	*fimH*	-2,1	0,007356	Manges et al., 2015
P22256	*E. coli* K12	Other processes	4-aminobutyrate aminotransferase GabT	Catalyzes the transfer of the amino group from gamma-aminobutyrate to alpha-ketoglutarate to yield succinic semialdehyde	*gabT*	-3,2	0,032568	Guzmán et al., 2015

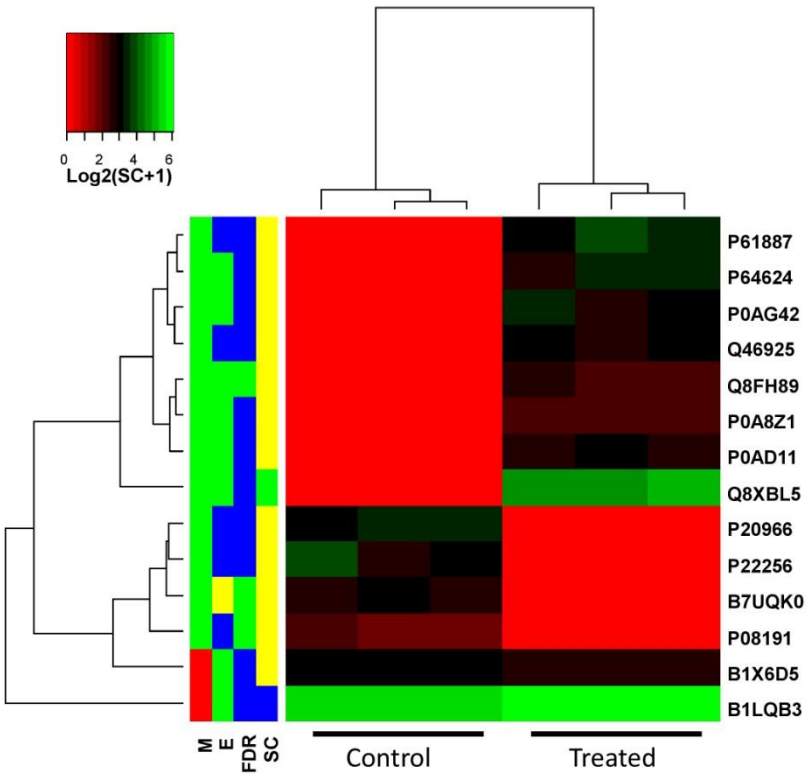

Figure 12.3. Heat map depicting significant regulated proteins after *p* value correction based on spectral counting (FDR-corrected *p* value ≤ 0.05). M depicts more (green) or less (red) than 2-fold regulated. Evidence group: E= 1 (blue), 2 (green), 3 (yellow). FDR for identification: blue <=0.001, green (0.001-0.01]. Spectral counts: [10-50) yellow, [50-100) green and >=100 blue.

Bacterial NAD^+-dependent DNA ligase (Q8XBL5) plays a critical role in DNA replication, recombination, and repair in all living organisms (Mills et al., 2011). Moreover, this protein was recently evaluated as a potential bacterial broad-spectrum drug target using adenosine analogs to inhibit the LigA activities of *E. coli*, *Haemophilus influenzae*, *Mycoplasma pneumoniae*, *Streptococcus pneumoniae*, and *Staphylococcus aureus* (Mills et al., 2011). In this study, LigA was the most up-regulated protein, reaching up to 4.7 log2 fold change when compared to the control group, which reinforces its likely bacterial survival response to the tetracycline-induced stress and potential as an effective drug target.

Glucose-1-phosphate thymidylyl transferase (P61887), which in this study was also found to be significantly up-regulated in the treated *E. coli*, is part in the L-rhamnose biosynthesis pathway (Blankenfeldt et al., 2000a). In turn, L-rahmnose is located in the cell wall of Gram negative and Gram positive bacteria, where is one of the important residues of O-antigen lipopolysaccharide, a key determinant for virulence and complement cascade mediated serum killing (Blankenfeldt et al., 2000a; Blankenfeldt et al., 2000b).

Hence, the up-regulation of such protein might be related with the increase in the pathogenicity of this environmental-borne strain when exposed to this antibiotic.

The up-regulation of a kinase (P0AG42), which is involved in the riboflavin biosynthesis pathway, is also relevant, considering that *tetX* encodes an unprecedent flavin-dependent monooxygenase that selectively hydroxylates this class of antibiotics, conferring resistance to all clinically relevant formulas, including tigecycline, a last resource tetracycline (Yang et al., 2004; Volkers et al., 2011). The up-regulation of this protein suggests a broader involvement of flavins in tetracycline-induced stress response (Yang et al., 2004).

Finally, the detection of the bacterial adhesion protein FimH (P08191) confirmed the pathogenicity of this environmental-borne *E. coli* and corroborated our previous molecular characterization showing a 93.1% chance of the isolate being a human pathogen. However, the adhesion type 1 fimbriae was 4.2-fold down-regulated in the tetracycline-challenged strain, which has been shown to result in the decrease of motility and adherence. Flagellum-mediated motility and chemotaxis have been suggested to contribute to virulence by enabling uropathogenic *E. coli* to escape host immune responses and disperse to new sites within the urinary tract. Thus, the decrease in motility in antibiotic challenged-isolates seems to decrease the global pathogenicity of the isolate (Snyder et al., 2005; Simms and Mobley, 2008a; Simms and Mobley, 2008b).

Overall the results indicate that *E. coli* responses to tetracycline are related to protein translation as well as metabolic regulation. Several metabolic proteins were differentially regulated, indicating a concerted activity of the bacteria to modulate the response against antibiotic exposure using its own metabolism. Five acquired antibiotic resistance genes were confirmed by genomic characterization and we concurrently identified a subset of these in the soluble fraction at the protein level (Table 12.2). The proteins penicillin TEM-135, the extended-spectrum β-lactamase CTX-M-1 and the dihydropteroate synthase Sul2 were identified but not found to be significant regulated in the soluble fraction upon tetracycline exposure. The efflux pumps TetA and TetB are membrane proteins and as expected not identified in the soluble fraction.

Identified proteins involved in virulence, antibiotic resistance and transfer of foreign DNA

Overall, considering the environmental origins of *E. coli* EcAmb278, it is important to highlight the proteins related with virulence, antibiotic resistance, and transfer of foreign DNA (Table 12.2). We were able to identify several proteins associated with antibiotic resistance in *E. coli* EcAmb278, such as β-lactam resistance mechanisms CTX-M β-

lactamase (P28585) and TEM (P62593), confirming previous results obtained and explored, using complementary DNA-based molecular methods (Jones-Dias et al., 2016).

The acquired (*sul2*) and chromosomal (*folP*) dihydropteroate synthase-encoding genes are associated with the different levels of resistance to the sulphonamides. FolP and Sul2 showed comparable abundance levels (iBAQ of 21.5 20.3, respectively), despite conferring resistance to the same class of antibiotics (van Hoek et al., 2011). Although acquired versions of FolP have been frequently described, in this study the location of *folP* gene was not explored.

We have also detected three of the proteins involved in the AcrAB-TolC and MdtEF-TolC multidrug efflux systems: AcrA (P0AE06), TolC (P02930) and MdtE (P37636). The drug substrate profile of *E. coli* AcrAB-TolC includes the antibiotics chloramphenicol, fluoroquinolones, fusidic acid, lipophilic β-lactam antibiotics, nalidixic acid, novobiocin, rifampin and tetracycline, as well as other compounds such as acriflavine, ethidium bromide, bile salts and short-chain fatty acids (Ruiz and Levy, 2014). Indeed, MdtE was among the most abundant proteins according to the calculated iBAQ values in this *E. coli*, in both conditions tested, which suggests an increased efflux in this strain. However, high-level resistance to tetracycline is normally displayed only when the AcrAB-TolC is associated to an additional tetracycline-specific resistance mechanism or a significant decrease in permeability (de Cristóbal et al., 2006).

Besides TolC, six other porins were identified (P0AA16, P0A917, P77747, P0A915, P0A910, and Q8XE41), which are components of permeability channels that allow passive diffusion of small molecules, like antibiotics, across the outer membrane.

It is known that, in *E. coli* strains, the down-regulation or loss of function due to amino acid substitutions in OmpC or OmpF promote resistance to some antibiotics, including tetracyclines (Delcour, 2009). In this study, although the down-regulation of those porins has not been detected, we cannot exclude the possibility of decreased permeability due to loss of function. Although the roles of OMPs and efflux channels are well established, it is not clear which signals are responsible for their specific activation. Moreover, OMPs play important roles in the adaptability, virulence and resistance of bacterial pathogens and the expression of some OMPs is regulated by the environment (Piras et al., 2012).

Overall, five other detected proteins have been associated with resistance to rifampicin (P0A903), tellurite (P25397), novobiocin and mecillinam (P0A9F5), and ampicillin (P0AD68, P10121) when mutated (Table 12.2).

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Table 12.2. Specific proteins identified in EcAmb278 that are involved in the processes of resistance, virulence and transfer of foreign DNA.

AccessionNumber	Protein	Protein functional description	Gene name	Evidence level	iBAQ
Antibiotic resistance					
P28585	CTX-M	Broad spectrum β-lactamase which confers resistance to penicillins, first, second and third and fourth-generation cephalosporins	*bla*	1	25.7
P62593	TEM	β-lactamases can confer resistance only to penicillins and first generation cephalosporins, or also to second and third generation cephalosporins, respectively	*bla*	1	21.6
P0AC11	Sul	Acquired dihydropteroate synthase intervenes in the folic acid pathway and confers resistance to sulphonamides by competing with the native bacterial dihydropteroate synthase.	*sul2*	1	21.5
P0AC13	FolP	Low level sulfonamide resistance occurs by mutations in the *folP* gene which encodedthe chromossomal dihydropteroate synthase.	*folP*	2	20.3
P0AE06, P02930	AcrA, TolC	AcrAB-TolC is a drug efflux protein complex with broad substrate specificity that uses the proton motive force to export substrates through the porin TolC, conferring resistance to a broad spectrum of antibiotics	*acr, tolC*	2, 1	21.6, 20.7
P37636	MdtE	Part of MdtEF-TolC multidrug efflux transport system, which confers resistance to various compounds	*mdtE*	3	19.2
P0AA16, P0A917, P77747, P0A915, P0A910,Q8XE41	OmpR, OmpX, OmpN, OmpW, OmpA, OmpC	Outer membrane proteins (also designated porins) that form channels to allow the passage of molecules through the outer membrane. Under-expression or aminoacid substitutions in these proteins are involved with resistance to many compounds	*ompR, ompX, ompN, ompW, ompA, ompC*	2, 2, 1, 1, 2, 3	23.1, 22.9, 17.3, 20.1, 24.2, 20.4
P0A903	BamC	Outer membrane protein involved in assembly and insertion of outer membrane β-barrel proteins (OMPs); strains with deleted *bamC*show	*nlpB*	1	20.2

		sensitivity to rifampicin			
P25397	TehB	Cytoplasmic methyltransferase that confers resistance to tellurite when expressed on a multicopy plasmid	*tehB*	1	20.0
P0A9F5	CysB	Mutations in *cysB* have been related with *E. coli* resistance to novobiocin and resistance to the β-lactam antibiotic mecillinam	*cysB*	2	19.9
P0AD68	FtsI	Involved in cell division and synthesis of peptidoglycan chains during cell cycle. When altered these penicillin-binding proteins mediate resistance to β-lactam antibiotics in Gram-positive and Gram negative bacteria	*ftsI*	2	5.5
P10121	FtsY		*ftsY*	1	21.3
Virulence, DNA transfer and others					
P24218	DLP12	Prophage integrase required for integration of the phage into the host genome	*intD*	1	NA
P0AF96	TabA	Toxin-antitoxin biofilm protein involved in biofilm formation and repression of fimbria genes	*tabA*	1	20.9
Q8FF56	RodZ	Gene essential for cell-to-cell plasmid transfer and DNA acquisition	*rodZ*	1	21.2
P32885	TraT	Tra system is associated with plasmid conjugation; responsible for preventing unproductive conjugation between bacteria carrying plasmids	*traT*	1	20.1
P03835	InsG	Involved in the transposition of the insertion sequence IS4, which is commonly associated with transfer of antibiotic resistance	*insG*	1	16.5
P05846	TnsE	Transposon Tn7 transposition protein; normally associated with class 2 integrons that carry antibiotic resistance gene cassettes	*tnsE*	1	NA
P05819, P02978 Q47502, P08083 P04419	Colicins B, E1, K, N, E2	Group of bacterial protein toxins produced by *E.coli* to reduce competition of other similar bacteria	*cba, cea, cka, cna, col*	1	18.9, 17.5,NA, NA, NA

In particular, the down-regulation of BamC (P0A903), a component of the β-barrel assembly machinery complex, responsible for recognition and assembly of outer membrane proteins (Omps) (Albrecht and Zeth, 2011), has been observed in *E. coli* isolates resistant to tetracycline and ampicillin, in proteomic studies performed in presence of those antibiotics (Xu et al., 2006).

The proteins involved in virulence and DNA transfer described in Table 12.2, highlight the pathogenicity of this environmental-borne isolate. Indeed, we have identified proteins related with the formation of biofilms, as well as the transfer, acquisition and recombination of DNA elements (P0AF96, Q8FF56, P32885, P03835, P05846, P24218) that are associated with the dissemination of well documented resistance mechanisms, such as IS*Aba1* (from IS*4* family) and *tnsE* (from class 2 integrons), among others (Ramírez et al., 2010; Olivares et al., 2013).

The production of bacteriocins in response to worsening environmental conditions is one mean of bacteria to outcompete other microorganisms, being effective against closely related *Enterobacteriaceae*. We detected five of these bacterial protein toxins, including colicin E1, which has already been described as a virulence factor in uropathogenic *E. coli* (Smajs et al., 2010; Petkovsek et al., 2012). In fact, bacteriocin production is an exclusive and important pathogenic feature of *E. coli* strains of clinical origin, particularly associated with complicated urinary tract infections (Petkovsek et al., 2012).

Globally, the analysis of the iBAQ values showed that CTX-M β-lactamase was the second most abundant protein and the most abundant among the different resistance mechanisms and virulence factors, showing a value of 25.7. However, CTX-M β-lactamase was not found to be significant regulated upon tetracycline treatment. Furthermore, tetracycline antibiotics have no structural relation with β-lactam antibiotics. Thus, we can conclude that CTX-M β-lactamase expression is not affected by tetracycline exposure. Recent evidence suggested that the preservation of this resistance mechanism imposes no or negligible fitness costs on *E. coli*, persisting without appropriate antibiotic selection (Fischer et al., 2014). The high abundance of CTX-M registered in the absence of an appropriate challenger, such as cefotaxime, corroborates these findings. In fact, the only additional acquired antibiotic resistance gene detected by proteomics - TEM β-lactamase - showed a lower iBAQ value (21.6). These mechanisms of resistance have already been widely described in human clinical settings, and it is particularly associated with the intensive production of food-producing animals (Bortolaia et al., 2010; Leverstein-van Hall et al., 2011; Hartmann et al., 2012; Jones-Dias et al., 2014). Overall, the direct application of antibiotic-contaminated manure or wastewater, and the indirect application of antibiotic-based phytopharmaceutical agents in crops might be contributing to the

maintenance if not the increase of resistance mechanisms in commensal microorganisms such as *E. coli*. In this proteome profiling of EcAmb278 upon tetracycline stress we investigated the soluble cell fraction, so that this new perspective could provide a broaden understanding of the metabolic cell responses of *E. coli* to a widely used antibiotic.

12.5. Conclusion

Although previous calls have been made to address antibiotic resistance in an environmental perspective (Olivares et al., 2013), it is important to highlight that the metabolic burden on a resistant pathogen is highly dependent on the bacterial microenvironment and the metabolic adaptations required for colonizing such a habitat (Bhargava and Collins, 2015). Considering that soils are persistently contaminated with resistant strains and that tetracyclines are still deliberately applied in crops, understanding the metabolic processes affected by antibiotic exposure of resistant strains is crucial.

The large scale quantitative proteome comparison of an environmental-borne *E. coli* challenged with tetracycline with the same strain without antibiotic exposure identified 8 up- and 4 down-regulated proteins upon tetracycline stress using a corrected *p* value threshold <0.05 and at least 2 fold regulation.

Our observed down regulation of proteins in the functional gene ontology categories "aerobic respiration" and "phosphate transport" and "ATPase activity" upon tetracycline treatment confirms previous observations based on metabolomics (Lobritz et al., 2015). These findings also concur with the findings of Lin and co-workers (Lin et al., 2014) which demonstrates down regulation of proteins involved in energy metabolism upon chlortetracycline treatment of *E. coli*. We additionally found that down regulation of a specific set of peptidoglycan-based cell wall proteins were replaced by up regulation of another set of peptidoglycan-based cell wall proteins upon tetracycline exposure. Furthermore, proteins unique for *E. coli* in the presence of tetracycline were enriched in gene ontology category manganese ion binding.

The microbial physiology shows that a single protein can act in a variety of cellular processes, including antibiotic resistance, although this may not be its primary role (Olivares et al., 2013). Thus, we highlight that resistance is not exclusively the result of a single altered protein, but rather a comprehensive and concerted metabolic process, as indicated by the proteome adjustments observed in the bacteria of this study.

In the end, investigating the relationships between bacterial metabolism and antibiotic susceptibility can help to uncover novel strategies for treating infections.

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Chapter 13.

General discussion and conclusions

It is now evident that antibiotic resistance is one of the major health concerns of the 21st century (Nolte, 2014). Despite the pressure caused by the misuse and abuse of antibiotics in human clinical settings, there are many actions associated with non-human environments that can influence the evolution of antibiotic resistance (Holmes et al., 2015). The interplay between different ecologies is especially important in this context. There are multiple links between the human, animal and environmental compartments that allow not only movement of bacteria, but also of mobile genetic elements and antibiotic molecules. Throughout this work we learned that bacteria recovered from very distinct habitats, such as agricultural soil, food from vegetable and animal origin, and animals of different sources, can harbor clinically important antibiotic resistant bacteria. The continuous flow of these elements between the different niches can reach concerning proportions and have serious consequences to humans and to the environment (Holmes et al., 2015).

The research conducted throughout this Ph.D. thesis enabled us to clarify specific issues related with the occurrence of mobile antibiotic resistance mechanisms in Gram negative bacteria recovered from non-human hosts: what are the main antibiotic resistance genes in non-human settings? How are they distributed among animals, soils and vegetables and how are they related with the genes that circulate in urban environments? How different anthropogenic actions contribute to the presence of antibiotic resistance? What are the main mobile genetic elements associated with the spread of specific antibiotic resistance genes in the above mentioned settings? Are they disseminated or is there an immediate risk for their dissemination?

These questions are answered in the research paper-based chapters (3 to 12), where about 1100 Gram negative isolates were studied in the scope of antibiotic resistance, after being collected from different sources in diverse regions of the country. Although a specialized discussion has been included in each manuscript-based chapter, the main findings will now be globally correlated and discussed in the present topic (Chapter 13, General discussion and conclusions).

Real time surveillance has an imperative role among the strategies implemented with the purpose of controlling the spread of antibiotic resistance. According with EARS-Net, many European countries showed significant increases in frequencies of antibiotic nonsusceptibility in the last years (EARS-Net, 2015). The 2015 European Food Safety Administration (EFSA, 2015) report on *Antimicrobial Resistance in Zoonotic and Indicator Bacteria from Humans, Animals and Food*, jointly analyzed by the European Center for

Disease Control and Prevention (ECDC), showed that the nonsusceptibility registered in isolates recovered from animals agrees with this trend (EFSA, 2015). In *Salmonella* from humans, high rates of isolates were resistant to ampicillin, tetracyclines and sulphonamides, while proportions of isolates nonsusceptible to third generation cephalosporins and fluoroquinolones generally remained low. In turn, for *Salmonella* and *Escherichia coli* isolates from fowl, pigs, cattle and meat, resistance to ampicillin, tetracyclines and sulphonamides was also commonly detected, while resistance to third generation cephalosporins was uncommon (EFSA, 2015). Either in human or animal isolates, the increasing antibiotic nonsusceptibility rates are a direct consequence of a raise in the number of mobile antibiotic resistance mechanisms (EFSA, 2011).

Despite recent studies focusing on mechanisms of resistance in food animals (Clemente et al., 2014; Gomes-Neves et al., 2014; Guerra et al., 2014; Kashoma et al., 2015; Skočková et al., 2015; Day et al., 2016), the scenario of plasmid-mediated antibiotic resistance in isolates from Portuguese veterinary settings is continuously shifting and deserves to be well understood. The numbers of plasmid-mediated quinolone resistance (PMQR), extended-spectrum β-lactamases (ESBL) and plasmid-mediated AmpC β-lactamases (PMAβ) have been increasing in isolates of animal origin throughout the years (EFSA, 2011). In food animals, the high consumption of fluoroquinolones (for instance, enrofloxacin in poultry) and third generation cephalosporins (for instance, ceftiofur in cattle), together with the lack of measures that moderate the spread of infectious diseases, have been appointed as the propelling forces (CIWF, 2011; EMA, 2015). Therefore, in Chapter 3 we have investigated the presence of PMQR determinants among *Salmonella* spp. and *E. coli* isolated from food animals and products. The study documents the occurrence of genetically non-related isolates carrying *qnrB2* (n = 3), *qnrB19* (n = 3), and *qnrS1* (n = 9) genes, associated with relevant genetic elements like *intI1*, IS*26* or even IS*CR1*, and enclosed in plasmids of different incompatibility groups. All but one isolate presented mutations in the quinolone resistance determining region (QRDR), which is consistent with the range of MIC values detected for fluoroquinolones. The majority of the PMQR-positive isolates were nonsusceptible to β-lactam antibiotics, which was justified by the presence of different β-lactamases. Thus, co-selection through the use of other antibiotics may occur once PMQR genes are embedded in plasmids harboring additional resistance genes (Vien et al., 2012; Economou and Gousia, 2015). Since their first discovery in 2002, PMQRs have emerged in humans and animals in Europe (Martínez-Martínez et al., 1998; Tran and Jacoby, 2002), as we corroborated with our study. However, despite the fact that fluoroquinolones have been used in food animals since the 80's, PMQR genes occur only occasionally in animal isolates, which suggests that PMQR-

positive isolates without additional mutations in the QRDR region are not selected during treatment of animals with fluoroquinolones. Considering the high use of fluoroquinolones in veterinary practice, long term surveillance is still needed for the monitoring of the future occurrence of PMQR genes worldwide (Veldman et al., 2011).

The levels of β-lactam nonsusceptibility, and particularly the existence of third generation cephalosporin nonsusceptible *E. coli* within the previously studied collection of isolates, directed us to perform a closer investigation in Chapter 4. The manuscript evaluates the importance of ESBL and PMAβ within a group of *E. coli* from food animals and determines the extent of their association with mobile genetic elements. CTX-M-1 was tightly associated with poultry, which corroborates the findings reporting the presence of CTX-M-1 in poultry from Switzerland, Germany, Tunisia and U.S.A., among others (Wittum et al., 2012; Grami et al., 2013; Belmar Campos et al., 2014; Zurfluh et al., 2014). This β-lactamase, together with other clinically relevant cephalosporin resistance determinants (CTX-M-14, SHV-12 and CMY-2), was associated to multiple sequence-type transferable plasmids that assured the dissemination of ESBLs and PMAβ between different animal husbandry units. Although ESBL/PMAβ-harboring isolates carried other plasmids, IncI1 seems to be responsible for this epidemic-like spread, which has already been cause for concern in poultry productions from other countries (Smith et al., 2015). Although *Enterobacteriaceae* with PMAβ is an important cause for cephalosporin and cephamycin nonsusceptibility, in this study, high-risk clones did not play a key position in the global dissemination of these enzymes. When Dierikx et al. (2013) analyzed broilers and broiler farmers, they identified $bla_{\text{CTX–M–1}}$ gene located on IncI1 plasmids, but in contrast to our study, these plasmids belonged mainly to ST7 (Dierikx et al., 2013). Similarly, in one Slovakian study, vertical transmission of third generation cephalosporin resistant *Enterobacteriaceae*, mainly CMY-2-producing *E. coli* was reported (Gregova et al., 2012). Considering that such cephalosporins are not the antibiotic of choice to treat poultry, evidence suggests that IncI1/ST3 plasmids harboring the $bla_{\text{CTX–M–1}}$ gene may be transmitted in the poultry industry without any significant selection pressure. Indeed, a recent study showed that, *in vitro*, the IncI1 plasmid carrying the $bla_{\text{CTX-M-1}}$ gene imposes no or negligible fitness costs on *E. coli*, and persists without antibiotic use (Fischer et al., 2014). Moreover, IncI1-driven CTX-M-1 is predominant in disease-associated isolates, which are also often from sequence types associated with human infections (Olsen et al., 2014). Intervention actions aiming to reduce the spread of CTX-M-1-producing *E. coli* should be materialized and the situation should be periodically re-assessed in the light of any new findings (Zurfluh et al., 2014).

Animals have the potential to act as reservoirs for a number of bacterial zoonotic infections, which might be transmitted to humans through direct contact or via the food chain (Marshall and Levy, 2011). In addition to food animals, companion animals, including dogs, cats and horses may also constitute potential reservoirs of PMQR-, ESBL- and PMAβ-encoding genes, as they often live in close contact with their owners, facilitating the transmission of bacteria (Ewers et al., 2012; Donati et al., 2014; Schmiedel et al., 2014). Wildlife and captive animals may also represent a source of antibiotic resistant *E. coli* isolates, among which we highlight the importance of the zoonotic transmission that is favored by the permanent contact between zoo animals, zookeepers and visitors in urban environments (Dobiasova et al., 2013; Guerrero-Ramos et al., 2016).

The data recovered from food animals and the findings of clinically important antibiotic resistance mechanisms in animals around the world, motivated us to study a collection of animals of different sources, mainly with regard to mobile fluoroquinolone and β-lactam resistance. Are the mechanisms circulating in animals from urban settings the same of animals from the food chain? In Chapter 5, the detection of antibiotic resistance mechanisms, such as PMQR and ESBL, lead the way to a wider genomic characterization of four specific *E. coli* isolates. Antibiotic susceptibility testing of the bacterial collection in which these isolates were included revealed a high number of non wild-type isolates for fluoroquinolones among poultry isolates recovered from *Salmonella enterica*. In turn, the frequency of non-wild-type *E. coli* to nalidixic acid and ciprofloxacin was higher in food-producing animals than in other animal groups. But it was among *E. coli* from companion and zoo animals that we have detected four PMQR and two ESBL determinants: two *qnrS1* were detected in isolates recovered from a dove and a dog and two $bla_{\text{CTX-M-15}}$ and *aac(6´)-Ib-cr* in isolates recovered from two different dolphins. The identification of many other genomic features showed us great genetic relatedness between the two *qnrS1*- and the two $bla_{\text{CTX-M-15}}$ and *aac(6')-Ib-cr*-harboring isolates. This data documented two main scenarios: the presence of the same strain in different hosts inhabiting isolated locations and the persistence of a single strain in a particular niche during a long period of time. Moreover, the ST131 CTX-M-15-producing *E. coli* constitutes one of the most successful high risk clones of all times: emerged during the early 2000s, spread extensively throughout the world, is responsible for the rapid increase of antibiotic resistance in *E. coli* and has even differentiated into sublineages (Mathers et al., 2015a). The worldwide dissemination of *E. coli* with CTX-M enzymes and PMQR determinants has been very efficient and involved health care settings, community, livestock, companion animals,

wildlife, and the environment (Mendonça et al., 2007; Hartmann et al., 2012; Fischer et al., 2014; Jamborova et al., 2015).

The detection of a ST131 H30-Rx *E. coli* in captive dolphins, which are permanently in contact with humans in urban habitats, encouraged the evaluation of its zoonotic potential. In fact, clinically relevant multidrug resistant *E. coli* isolates have been on the rise for years (Coque et al., 2008). Initially restricted to clinical contexts, recent findings suggest that their prevalence in non-clinical settings is maintained by the constant exchange of isolates (Mathers et al., 2015a). Thus, in Chapter 6 we explored the genetic relatedness between one of the ST131 CTX-M-15–producing *E. coli* isolated from a captive dolphin, and a collection of clinical isolates recovered during the same time span, from five Portuguese health care facilities. The study illustrated the clonality between a set of human clinical isolates and the animal isolate, showing common antibiotic resistance genes (mainly $bla_{\text{CTX-M-15}}$ and *aac(6')-Ib-cr*) and plasmids (IncF). It appears that the isolates evaluated in this study have been submitted to the same evolutionary genetic events, which ultimately led to the establishment of the same allelic diversity pattern (ST131 fimH30-Rx). There is increasing evidence for the transmission of antibiotic resistant bacteria between wildlife, humans, companion animals, food animals, farm animals and the wider environment (Antunes et al., 2004; Ewers et al., 2014; Wang et al., 2015a). This study confirms the zoonotic potential of the isolate and alerts for the possibility of interspecies transmission of an international high-risk clone. However, the direction of transfer between animals and humans is still uncertain: it probably goes both ways and involves other critical compartments as mediators (Dantas and Sommer, 2014). Globally, the results strengthen the importance of One Health approach, which recognizes that the health of humans is connected with the health of animals and the environment (https://www.onehealthcommission.org). This vision involves improved communication, cooperation, and collaboration among the many disciplines and organizations with important roles to play at the intersection of human, animal, and environmental health (Lammie and Hughes, 2016).

It is not surprising that soil is a major reservoir of antibiotic resistance because it is likely to be as natural, widespread and ancient as antibiotic production itself (Nesme and Simonet, 2015). Despite the occurrence of naturally produced antibiotics and chromosomally harboured antibiotic resistance genes there have been reports of soil contamination with biopollutants (Berendonk et al., 2015). Indeed, manure and untreated wastewater with origin in animal husbandry facilities can be sources of antibiotic resistance (Wachtel et al., 2002; Islam et al., 2004a; Islam et al., 2004b). In Chapter 7, we

evaluated the soil population of culturable Gram negative bacteria recovered from agricultural soils and assessed the presence of acquired resistance to β-lactam antibiotics given by ESBLs, PMAβ and carbapenemases from molecular classes A, B and D. We went further on and also analysed the influence of different agricultural practices regarding the emergence of antibiotic resistance in agricultural soils. The widespread presence of multidrug resistant Gram negative isolates was noticed in soils from intensive, extensive and organic agricultural practices. When the nonsusceptibility levels of the different antibiotics were separately analysed, high rates of resistance were noticed in intensive soils for norfloxacin, streptomycin and tetracycline. Exposure to intensive agricultural practices was even considered a risk factor for nonsusceptibility to many antibiotics, multidrug resistance and production of ESBL. To date, a few studies analysed agricultural soils with regard to antibiotic resistance bacteria (Hartmann et al., 2012; Merchant et al., 2012; Blaak et al., 2015; Gatica et al., 2015) and none have characterized the isolates to this extent. We identified a multiple sequence-type IncI1-driven spread of penicillinases (*bla*$_{\text{TEM-1}}$, *bla*$_{\text{TEM-135}}$), ESBL (*bla*$_{\text{SHV-12}}$ and *bla*$_{\text{CTX-M-1}}$) and PMAβ (*bla*$_{\text{CMY-2}}$). The slight predominance of pMLST ST3 IncI1-bearing CTX-M-1 and the detection of ICE*cp1*-*bla*$_{\text{CTX-M-1}}$-*orf477* fragment, regardless of the genetic origin of the isolates, suggested horizontal spread of transferable IncI1 plasmids. Moreover, the detection of isolates with similar profiles in intensive agricultural soils (that benefit from manure amendments and application of pesticides or other chemical enhancers) and in samples from poultry (Chapters 4 and 7) suggests a widespread distribution of ST3 IncI1-bearing CTX-M-1 in intensive agricultural settings. To date, CTX-M-1 was described in isolates from soil in only one occasion but it was associated with clonal spread within a cattle farm (Hartmann et al., 2012). This can be considered a secondary route for the transmission of IncI1-harboring *bla*$_{\text{CTX-M-1}}$, since horizontal gene transfer is their main via of dissemination.

The evaluation of the extent of such clinically relevant antibiotic resistance genes in the food chain is a pertinent assignment and the assessment of fresh produce as supplementary carriers of antibiotic resistant bacteria has already started (Berger et al., 2010; Ruimy et al., 2010; van Hoek et al., 2015). In fact, Ruimy et al. (2010) from France has already declared that organic and conventional fruits and vegetables contained equivalent counts of Gram negative bacteria expressing antibiotic resistance. However, considering our previous experience with agricultural soils from Chapter 7 we hypothesized that Portugal might have a different scenario. Indeed, conventionally produced fruits and vegetables, which are usually grown in intensive farming settings, could be getting contaminated with clinically relevant acquired resistance genes or mobile genetic elements. The main objectives of this new study were to assess the information on

antibiotic resistance bacteria gathered from organically and conventionally grown fruits and vegetables produced and marketed in Portugal.

In fact, in Chapter 8 we showed that both organic and conventional fresh produce harboured antibiotic resistant Gram negative isolates, of which many were human commensals and opportunistic pathogens. For instance, multidrug resistant ST15 *Klebsiella pneumoniae*, detected in this study in organically-produced strawberries has been associated with nosocomial infections and outbreaks, and represents a major clone among CTX-M-15–producing isolates (Hu et al., 2013; Markovska et al., 2015). The molecular study of mobile antibiotic resistance, here initiated with the detection of class 1, 2 and 3 integrase-encoding genes, identified eleven antibiotic resistant opportunistic pathogens (e.g. *Enterobacter cloacae*, *E. coli* and *Morganella morganii*) and environmental species (e.g. *Raoultella ornithinolytica* and *Pseudomonas putida*) as integron producers (Marshall et al., 2009). Genomic characterization of these isolates showed that they carried an impressive number of clinically relevant acquired antibiotic resistance genes, such as *mcr-1*, *qnrA1*, *qnrA1*, $bla_{\text{GES-11}}$, *mphA* and *oqxAB*. Moreover, the study of the architecture of the integrons themselves led to the identification of different promoter regions (Pw, PH1, Ps, $PW_{\text{TNG-10}}$ and P2), diverse gene cassette arrays (containing *drfA*, *dfrB*, *aadA*, *cmlA, estX, sat* and bla_{GES}) and association with multiple transposable elements (e.g. Tn*402*, Tn*7*, *ISCR*1, Tn*2**, IS*26*, IS*1326* and IS*3*), reinforcing the ability of integrons to adapt (Cambray et al., 2010). Moreover, we showed that the processes by which mobile genetic elements disseminate antibiotic resistance genes are discrete, and may not cause evident high levels of resistance. Recent studies showed that bacterial exposure to specific antibiotics can trigger an SOS response that, in turn, is able to activate integrase-mediated recombination. Such connection provides bacteria with a remarkable antibiotic-induced mechanism for gene acquisition, reorganization and excision, which can be allied to the dispersal ability of transposable elements (Guerin et al., 2009; Cambray et al., 2011). The detection of integrons of three different classes, convertible and transferable "on demand", reinforce the mobilization potential of antibiotic resistance in Gram negative bacteria and their rapid escalation in the food chain (Guerin et al., 2009).

The detection of the plasmid-mediated colistin resistance *mcr-1* gene in a class 2 integron-producing *E. coli* recovered from a conventionally produced lettuce, confirmed that fresh produce are deeply involved in the dissemination dynamic of acquired resistance genes, just as animals and humans (Hasman et al., 2015; Stoesser et al., 2016; Yao et al., 2016). These findings are specially concerning because the majority of these food items are frequently consumed raw. Indeed, colistin is the only therapeutic option available for the

treatment of many infections caused by multidrug resistant *K. pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Bialvaei and Samadi Kafil, 2015; Liu et al., 2016). The first detection of *mcr-1* gene in fresh food from vegetable origin showed the importance of analyzing the genetic environment in which the gene was enclosed. Thus, in Chapter 9 we reported the epidemiological and genetic background of the multidrug resistant INSLAli25 *E. coli* isolate carrying *mcr-1*. Although few reports on *mcr-1* exist so far, the described genetic contexts for this gene show a remarkable molecular and epidemiological diversity. In this study, only a 2.6Kb fragment near *mcr-1* showed homology with previously described *mcr-1*-encoding regions. The remaining genetic surroundings of *mcr-1* in *E. coli* INSali25 had no resemblance with DNA of other isolates: the upstream region of the gene harboured a partial Tn*3* and the downstream region showed >99% identity with other genomic region from a MCR-1-harbouring *S. enterica* recovered from a food product, also in Portugal (Tse and Yuen, 2016). The 2013 advice of the European Medicines Agency (EMA) recommended maintaining the use of colistin in veterinary medicine for the treatment of infected animals, removing all indications for prophylactic use. Considering the latest developments in the field, the agency has been encouraged to update the advice, which is now under construction (EMA, 2016). In 2013, colistin was already a last resource antibiotic for the treatment of serious human infections (Bialvaei and Samadi Kafil, 2015). Thus, now, more than ever, it is urgent to alter the restrictions to colistin use, and to develop new and alternative antibiotic molecules.

As we documented in Chapters 3 to 9, *E. coli* is an obvious example of a silent pathogen (because it is often commensal) that can harbor transferable antibiotic resistance mechanisms, occurring in multiple hosts and that can easily circulate throughout the food chain (Veldman et al., 2011). With this agent as a major opportunistic pathogen in poultry and in the environment, and with a potential for zoonotic transfer to human beings, *E. coli* still represents one of the major microbiological risks to human health (Olsen et al., 2014).

Thankfully, despite the current increase in the number of mobile carbapenemases in humans around the world (Hawkey, 2015), including in Portugal (Manageiro et al., 2014; Manageiro et al., 2015a), their occurrence was not noticed in any of the bacterial collections studied in the course of this thesis. In order for this to be maintained, constant monitoring should be performed in bacterial isolates of different origins, and carbapenem use must continue to be restricted to complicated infectious caused by multidrug resistant microorganisms (Woolhouse et al., 2015).

In this thesis (Chapter 3 to 9) the widespread distribution of antibiotic resistance genes was associated with mobile genetic elements. For instance, PMQR-, ESBL- and PMAβ-encoding genes were associated with insertion sequences, integrons, transposons or highly promiscuous plasmids. It is widely accepted that horizontal gene transfer drives this gene flow (Mathers et al., 2015b). In fact, many studies have documented that horizontal gene transfer is a highly efficient mechanism for the transmission of genes between bacteria that can be affected by external factors (Stecher et al., 2012; Fischer et al., 2014). Throughout this thesis, conjugative plasmids from many incompatibility groups were associated with the transference of antibiotic resistance. For instance, IncI1 plasmids, mainly associated with bla_{ESBL}, were detected in different but interconnected compartments, such as animal husbandry units and intensive agricultural soils, which are likely to be related. As documented by Fischer et al. (2014), the efficiency of plasmid transference may be related with the absence of fitness costs for the host, while maintaining the eventual benefits of co-selection of other antibiotic resistance genes. Plasmids consist of one of the most complex and advantageous mobile genetic elements and, because of their role in the spread of antibiotic resistance they are also one of the most clinically relevant (Fernandez-Lopez and de la Cruz, 2014). The molecular characterization of *E. coli* and other isolates showed complex arrangements of mobile genetic elements, where more than one of these structures was physically associated.

In this work, antibiotic resistance genes were frequently flanked by insertion sequences or complete transposons, which, in turn, were harboured by isolates that possessed other mobile genetic elements, such as integrons. Indeed, specific cases such as the new class 2 integron in Chapter 4 retained our attention. This example illustrated the complexity of genetic events occurring within a single mobile element that enclosed multiple antibiotic resistance genes. The integron sequence resembled the classic In*2-4*, except within the recombination site where the cassette array that contained it was preceded by a complete IS*10* that resulted in the disruption of the original *attI2*, and in the reconstruction of a second *attI2*. Interactions within multidrug resistance regions were also conspicuous in class 1, 2 and 3 integrons recovered from Gram negative bacteria from fresh food of vegetable origin. Although integron gene cassettes were mostly dominated by *dfr* and *aadA* genes, their association with transposable elements such Tn*402* and Tn*7*, among others, underscored their support in genetic recombination events, suggesting an increased ability to move. Reported as widely distributed in bacteria, the mobility of integrons has been considered to be a major concern for the spread antibiotic resistance in clinical settings (Escudero et al., 2015). The detection of mobile resistance integrons in local food of vegetable origin has now aggravated this distress. The integron is a dominant

system which, by capturing, accumulating and rearranging new functions, confers bacteria rapid adaptation ability in changing environments. Furthermore, cassette and integrase expression are also highly controlled by host regulatory networks and bacterial stress response. This close association with the host makes the integron a genetically stable structure, granting the bacteria a highly adaptive system (Cambray et al., 2010; Escudero et al., 2015).

Once efficient mobile genetic elements are acquired by pathogenic agents, the clonal spread is a suitable mechanism for the perpetuation of resistance, as described in Chapters 5 and 6. Thus, one of the possible solutions to face antibiotic resistance might be related with blocking the transference of genes between pathogenic agents (Forsberg et al., 2014). The clustered regularly interspaced short palindromic repeats-associated protein 9 (CRISPR/Cas9) stands out as the ultimate opportunity to achieve such a goal (Horvath and Barrangou, 2010). Through the phage-mediated delivery of a functional CRISPR-Cas system into the genome of antibiotic resistant bacteria, the simultaneous elimination of antibiotic resistance plasmids and the genetically modified lytic phages that were transporting the system is likely to be achieved. Indeed, it was recently shown how it is possible to program lytic phages to kill only antibiotic resistant bacteria, while protecting antibiotic sensitized bacteria (Yosef et al., 2015).

Horizontal gene transfer is so insidious in prokaryotes that the classical definition of a unique genome is not suitable to describe the bacterial genomic mosaicism (Koonin et al., 2001; Vos et al., 2015). Bacterial species are characterized by a common core genome, shared by all bacteria of a single species, and by a larger pan-genome, that bears the genomic diversity of a taxon and is comprised of the core and accessory genomes (Vos et al., 2015). Most of the adaptation potential is present in this variable gene group, and much of the diversity found in the pan-genome is a result of horizontal gene transfer (Fernandez-Lopez and de la Cruz, 2014). The recent burst in *omic* technologies provides us with the opportunity to look into the bacterial pan-genome in a single glance. With the dawn of next generation sequencing technologies and their application in understanding multidrug resistant microorganisms, it has become urgent to analyze not only hazardous bacterial isolates and strains, but also complex environmental samples with unknown microbiological content (Xavier et al., 2016).

Hence, we were decided to take advantage of this late generation techniques to explore antibiotic resistance in single bacterial isolates. Following recent improvements in sequencing technologies, whole-genome sequencing (WGS) is in the run to become an

essential tool in the control of antibiotic resistance. Besides current applications in surveillance studies, these techniques have already found numerous purposes in infection control and diagnostics, either in the identification of outbreaks or in the detection of antibiotic resistance (Joensen et al., 2014). In Chapter 10 we aimed to explore and characterize the molecular support of multidrug resistance and pathogenicity of an avian *M. morganii* isolate. This microorganism is known as a common commensal bacteria and as an opportunistic pathogen that has been sporadically implicated in infections of humans or animals (O'Hara et al., 2000). In this study, we learned that its 4.3Mbp draft genome contained multiple indicators of a successful pathogen, including an assortment of antibiotic resistance genes that guaranteed resistance to aminoglycosides (*aadA1y*, *aph(3')-Ic*, and *strA-strB*), β-lactam (bla_{OXA-1}), fluoroquinolones (*qnrD1*, *aac(6')-Ib-cr*), phenicols (*catA2 and catB3*), rifampicin (*arr-2*) sulphonamides (*sul2*), trimethoprim (*dfrA1*), tetracycline (*tetY)*, and streptothricin (*sat2*). In addition, the isolate harboured several pathogenicity factors and mobile genetic elements that confirmed the inherent risk of animal-to-animal or animal-to-human transmission. The *qnrD* gene, now denominated *qnrD1* due to the report of a second variant of the gene (Abgottspon et al., 2014), was enclosed in a small nonconjugative plasmid (8,449bp), just like other such PMQRs previously reported in animals in China (Zhang et al., 2013). Although *qnrD* gene is a relatively rare antibiotic resistance gene, it is often described in members of the *Proteeae* family, regardless of their origin (Mazzariol et al., 2012; Zhang et al., 2013; Nasri Yaiche et al., 2014). In one of his studies, Guillard et al. (2014) looked for homologous plasmids in reference *qnrD*-negative *Proteeae* strains and found a 48% matching plasmid in *Providencia vermicola*. In the previously detected *qnrD*-harboring plasmids, this gene has been located within a mobile region showing similarities with areas of *P. vermicola* plasmid, suggesting that these small nonconjugative plasmids might be the product of a recombination between an unknown *qnrD*-bearing region and a native plasmid from *Proteeae*. Indeed, in our study, the presence of IS*26* inverted repeats also suggested the occurrence of a more recent genetic recombination event within the *qnrD*-encoding plasmid, which corroborates the previously reported data (Guillard et al., 2014).

The occurrence of opportunistic pathogens in poultry flocks, which are known to be abusively treated with antibiotics, can favor the acquisition of antibiotic resistance genes and transform innocuous commensal bacteria in treacherous pathogenic agents (EFSA, 2015; EMA, 2015). For this reason, we also characterized by WGS one of the *E. coli* isolates recovered from a broiler in the scope of Chapter 11. Isolate *E. coli* INSLA289 ST57 was selected as a good candidate to be massively characterized, due to the production of the already mentioned hybrid IS*10*-disrupted In*2-4* class 2 integron. The presence of this

element suggested the occurrence of genetic recombination events taking place in the core or accessory genome of the isolate (Ramírez et al., 2010). Besides this mobile element, the isolate showed a 91.4% probability of being a human pathogen, and harboured 12 different mobile antibiotic resistance genes. Together, they were sufficient to confer resistance to penicillins, third generation cephalosporins, aminoglycosides, sulphonamides, trimethoprim, tetracycline and streptothricin. Fluoroquinolone resistance was assured by QRDR alterations leaving no main veterinary therapeutic options available to be prescribed (EMA, 2015). Cefotaxime resistant *E. coli* ST57 has already been detected in broilers, cats, dogs and healthy humans in Tunisia (Ben Sallem et al., 2012; Ben Sallem et al., 2014), fecal samples of calves in the Netherlands (Hordijk et al., 2013) and healthy dogs in Mexico (Rocha-Gracia et al., 2015). Although today's human Gram negative infections are mainly dominated by pathogenic agents such as *K. pneumoniae*, community and environmental settings are mostly dominated by opportunistic bacteria, such as *E. coli* or *M. morganii* that can quietly gather genetic factors necessary to emerge as successful opportunistic pathogens. This crossover phenomenon that arises because of the nature of the species, but mostly because of the physiological state of the host, is deeply related with defining when microorganisms act as commensals and when they act as pathogens (Marshall et al., 2009) (Figure 13.1).

The interactions that occur between a bacterial cell and its surroundings (either the host or the environment where the microorganism is inserted) may be crucial to define a metabolic response and a role as a pathogenic agent (Baer and Millar, 2015). For instance, microorganisms that inhabit the soil are gifted with metabolic skills that allow them to survive their competitors and any biological or chemical challenge imposed by that habitat. Globally, when a bacteria is challenged with a stressor, such as an antibiotic, any cellular alterations necessary to fight back are reflected in their proteome, which can change in response to any external environmental factor (Burchmore, 2014).

Thus, in Chapter 12, we submitted an antibiotic resistant *E. coli* isolate to tetracycline, which was used as an external stressor. This isolate was previously recovered from an intensive agricultural soil in the scope of Chapter 7. We aimed to understand the global metabolic changes that may occur in a typical commensal bacterial such as *E. coli*, when faced with a high dosage of antibiotics, just as it happens when treating agricultural land with phytopharmaceutical products. It has been suggested that alterations in energy metabolism could constitute a relevant bacterial survival mechanism against antibiotic exposure (Lin et al., 2014). Our results support this proposition since we observed

enrichment of protein groups such as “aerobic respiration”, “phosphate transport” and “ATPase activity” in the absence of tetracycline.

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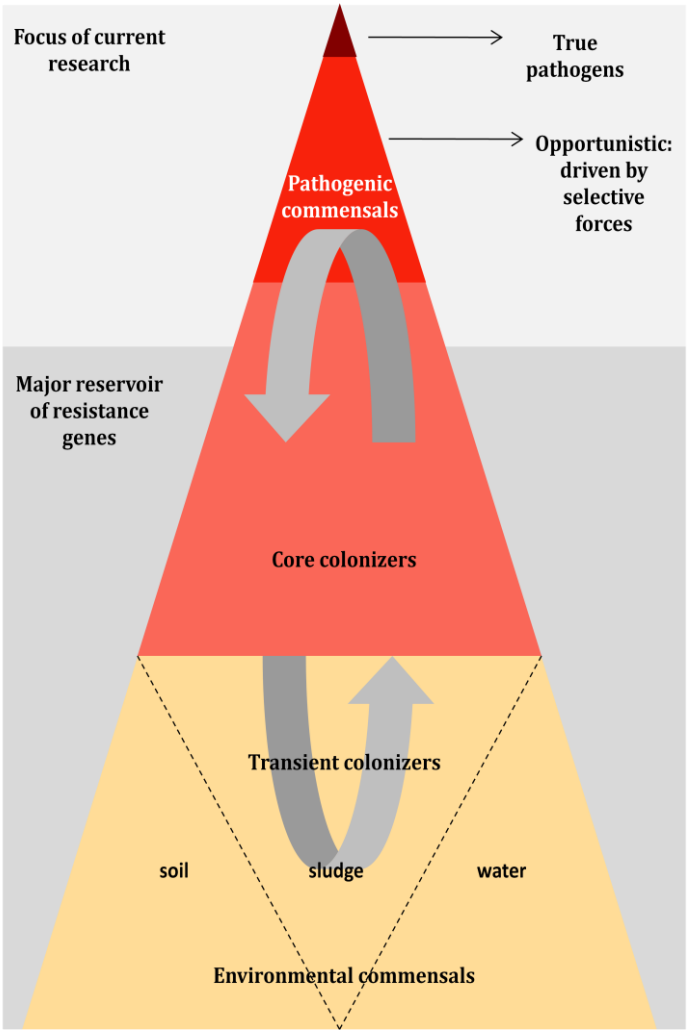

Figure 13.1. Hierarchy of commensal bacteria (adapted from Marshall et al., 2009)

We additionally found that down regulation of a specific set of peptidoglycan-based cell wall proteins were replaced by up regulation of another set of peptidoglycan-based cell wall proteins upon tetracycline exposure. Proteins unique for *E. coli* in the presence of tetracycline were enriched in gene ontology category “manganese ion binding”. We found out that a set of 12 proteins, distributed among biological processes such as DNA replication/repair, transcription, virulence or intracellular trafficking and secretion, were significantly regulated by more than 2-fold change. Furthermore, we also identified the presence of proteins that were not regulated but are deeply involved in processes of virulence, antibiotic resistance and transfer of foreign DNA, and that are significant to the definition of an *E. coli* as a pathogenic agent. The physiology of this *E. coli* isolate also

showed that a single protein can act in a variety of cellular processes, including antibiotic resistance, although this may not be its primary role, as already suggested in other studies (Olivares et al., 2013). The use of this type of proteomic approach for the identification of proteins that might be related with the response to antibiotic exposure represents an important achievement in determining the metabolic pathways that might be associated with antimicrobial activity and resistance (Vranakis et al., 2014; Franzosa et al., 2015).

The work performed in this thesis included a variety of reservoirs and techniques. By developing vigilant epidemiological studies carried out in different backgrounds, and by exploring them as deeply as possible, with resource to varied culture based methods and massive *omic* approaches, we were able to identify the major bacterial species and antibiotic resistance genes circulating in different non-human environments in Portugal.

Resistance genes and the genetic arrays that mobilize them into multidrug resistant bacteria of clinical significance are likely to have their origins in completely unrelated parts of the bacterial biosphere (Djordjevic et al., 2013). Once they become mobile trough the association with complex but efficient genetic elements they may turn into biopollutants that are then spread upon inadequate human actions and, eventually, appropriate antibiotic selection (Cantas et al., 2013). More attention needs to be paid to the way that humans involuntary supply and select mobile elements that enhance the flow of resistance between remotely connected ecosystems (Djordjevic et al., 2013). In fact, the challenge of tackling antibiotic resistance has a variety of similarities with another 21st century crisis: climate change. Among other parallels, both antibiotic resistance and climate change are natural processes operating on a global scale that human activity has severely influenced during the last 100 years (Woolhouse et al., 2015). Curiously, a worldwide significant decrease in livestock production and in the consumption of meat, already recommended by the World Health Organization due to the greenhouse gas emissions and other direct health related-problems, would also considerably benefit the fight against antibiotic resistance. Attention needs to be paid to those bacteria that link human, animal and environmental ecosystems, where many antibiotic resistant commensal bacteria have an important role.

The current *omic* era is offering the scientific community a chance to understand how resistance genes are mobilized, and how to contain this mobilization. In the long term, these holistic approaches constitute the best opportunity to better manage what is one of the main health problems of the world.

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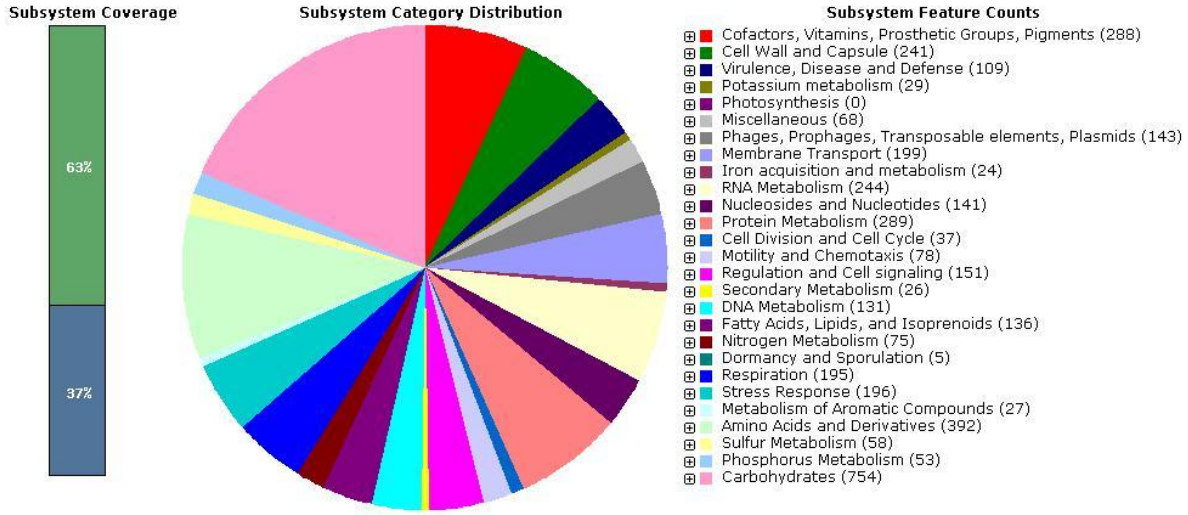
Supplemental material

A



B

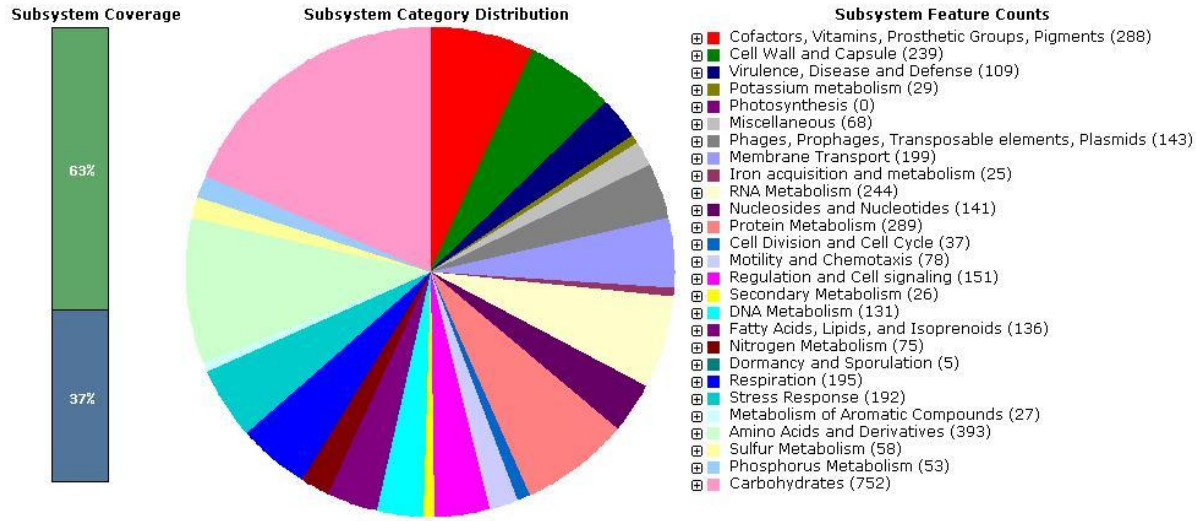
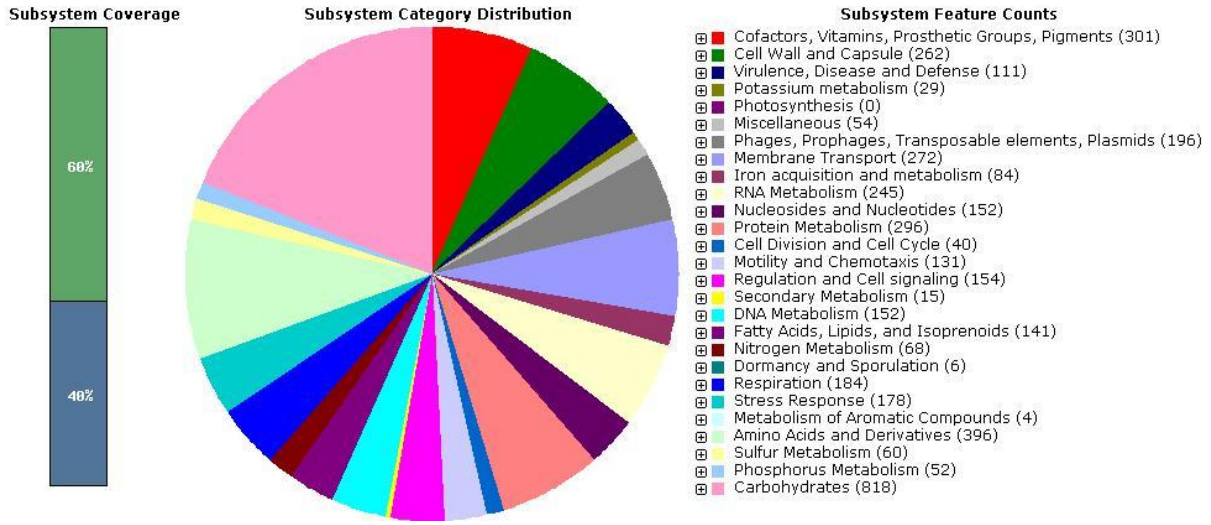


Figure S5.1. Representation of relative abundance of each subsystem category in isolates LV46221 (A) and LV46743 (B).

A



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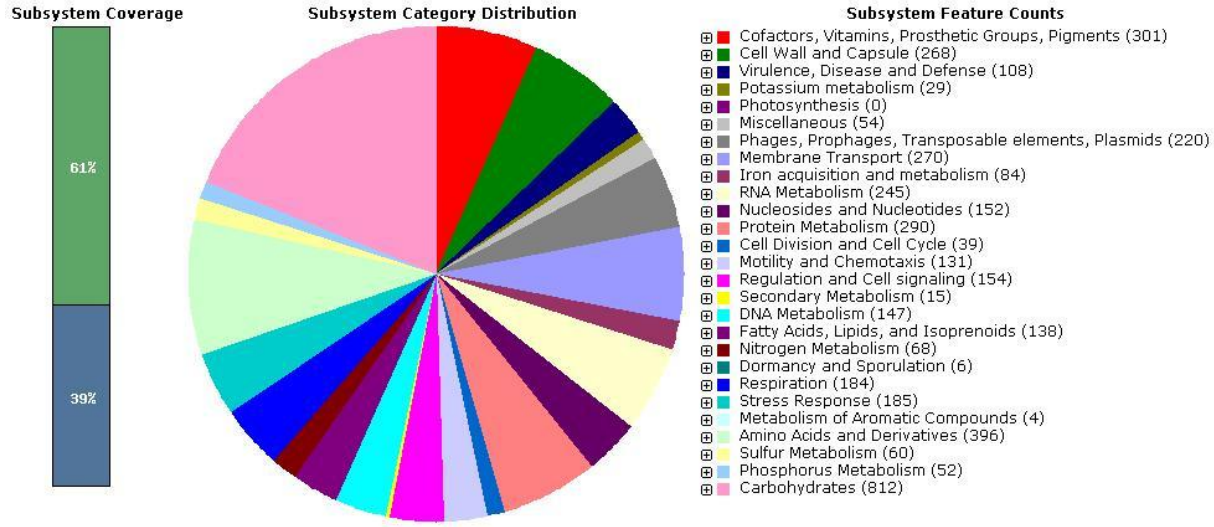

Figure S5.2. Representation of relative abundance of each subsystem category in isolates LV36464 (A) and LV27950 (B).

Table S5.1. Representation of intact phage regions detected in the draft genome of LV46221, LV46743, LV36464 and LV27950.

Phage regions	Region length (Kb)	Score	Number of coding sequences	Accession number
LV46221				
1	30.4	100	36	NC_003315
2	19.4	100	23	NC_016158
3	14.7	100	17	NC_001416
4	58.9	150	67	NC_021857
5	54.1	150	50	NC_010463
6	37.9	150	39	NC_001416
7	90.6	150	133	NC_001416
LV46743				
1	10.4	96	12	NC_001609
2	19	100	21	NC_001416
3	24.7	100	35	NC_010463
4	18.6	110	28	NC_028943
5	54.5	100	37	NC_022747
6	48.4	150	56	NC_021857
7	21.9	130	29	NC_016158
8	43	150	44	NC_001416
9	70.3	150	88	NC_001416
LV36464				
1	33.9	150	46	NC_001895
2	46.8	120	34	NC_026014
3	36.8	93	54	NC_009237
4	31.8	150	36	NC_001416
5	49.4	150	61	NC_019522
6	17.4	140	24	NC_004813
7	44.7	150	38	NC_004813
8	51.1	150	88	NC_019716
LV27950				
1	68.5	150	80	NC_019522
2	34.7	150	26	NC_019716
3	33.9	150	44	NC_022750
4	40.6	120	32	NC_026014
5	34.7	150	46	NC_005882
6	25.3	150	29	NC_004813
7	25.2	110	38	NC_001416
8	20.6	150	30	NC_001416
9	58.3	150	90	NC_019716
10	86.1	150	169	NC_001416

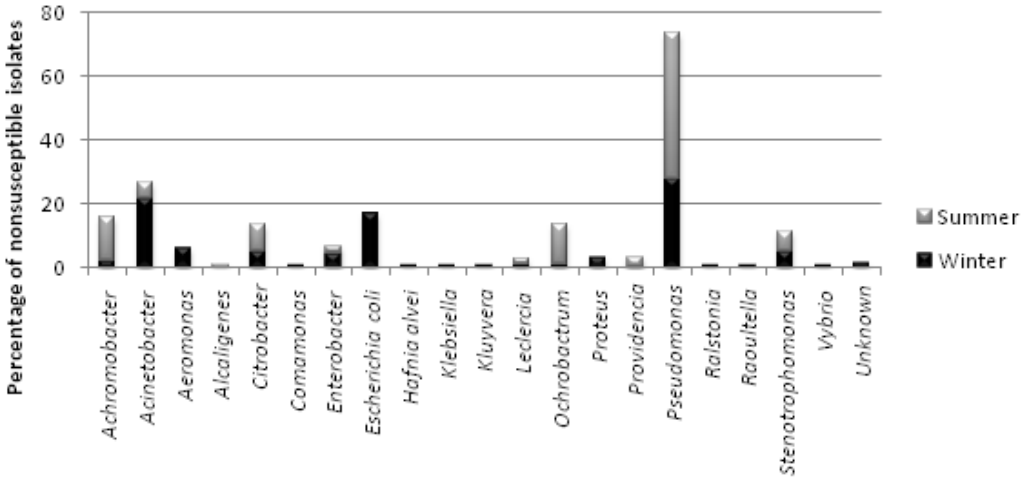

Figure S7.1. Percentage of bacteria nonsusceptible to amoxicillin, cefotaxime and/or imipenem (n=232) belonging to different genera identified in the studied soils by season. Winter, black; Summer, grey.

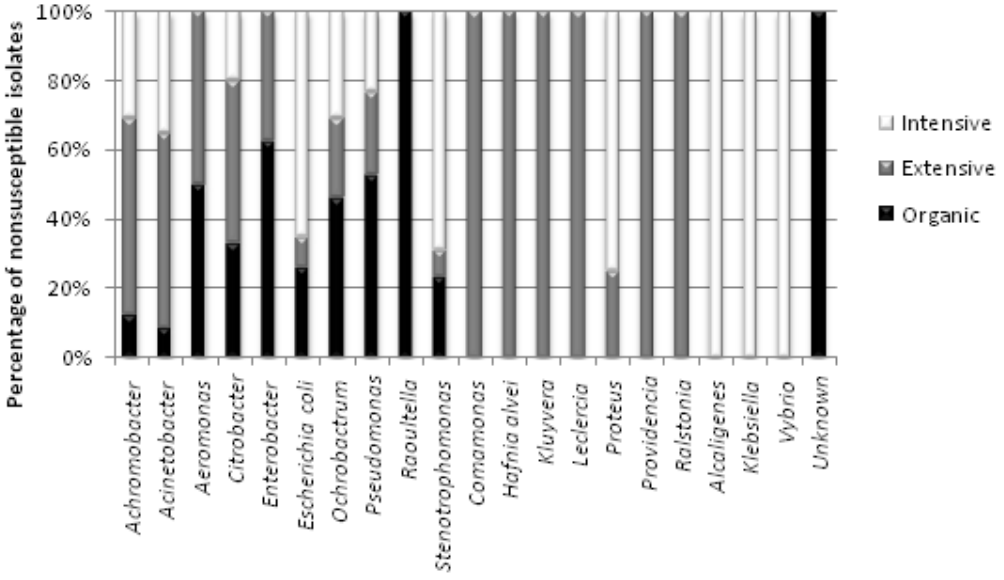

Figure S7.2. Percentage of bacteria nonsusceptible to amoxicillin, cefotaxime and/or imipenem belonging to different genera identified in the studied soils by type of agricultural practice (n=232). Intensive, white; Extensive, grey; Organic, black.

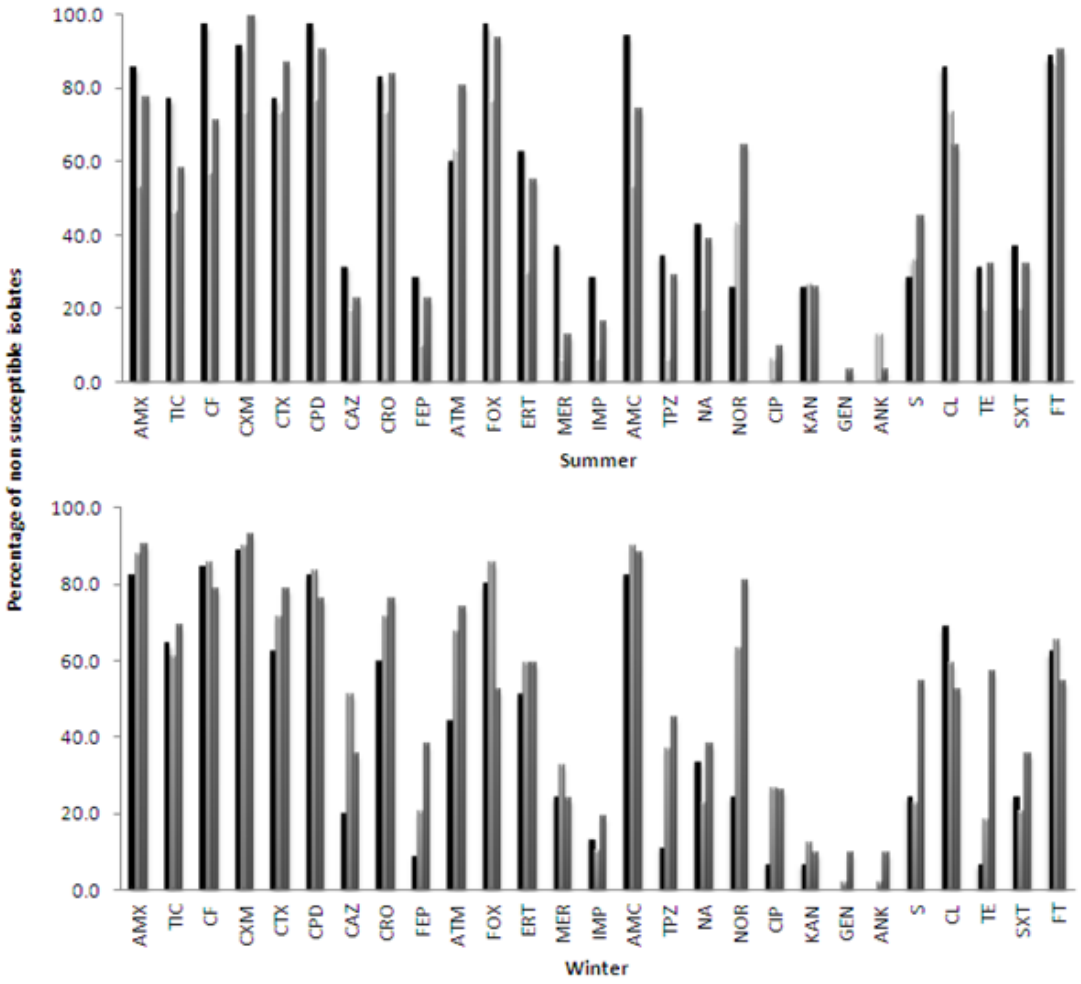

Figure S7.3. The antibiotic resistance profiles by percentage of nonsusceptible bacteria (n=232) according to the agricultural practice and sample season for 27 antibiotics of seven different classes, after previous selection with amoxicillin, cefotaxime and/or imipenem. Intensive, dark grey; Extensive, light grey; Organic, black.
AMX, amoxicillin; TIC, ticarcillin; CF, cephalotin; CXM, cefuroxime; CTX, cefotaxime; CPD, cefpodoxime; CAZ, ceftazidime; CRO, ceftriaxone; FEP, cefepime; ATM, aztreonam; FOX, cefoxitin; ERT, ertapenem; MER, meropenem; IMP, imipenem; AMC, amoxicillin plus clavulanic acid; TPZ, ticarcillin plus tazobactam; NA, nalidixic acid; NOR, norfloxacin; CIP, ciprofloxacin; KAN, kanamycin; GEN, gentamicin; ANK, amikacin; S, streptomycin; CL, chloramphenicol; TE, tetracycline; SXT, sulfamethoxazole; FT, nitrofurantoin.

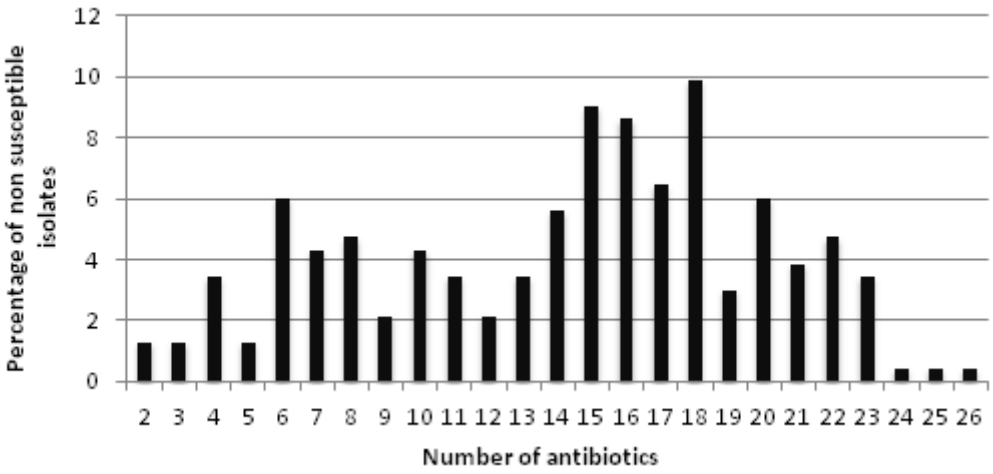

Figure S7.4. Percentage of 232 nonsusceptible bacteria to two or more antibiotics from different classes.

Table S8.1. Odds ratios (OR) and 95% confidence intervals (CI) from the analysis of negative and positive correlations for Gram negative nonsusceptible bacteria detected in conventionally and organically produced fruits and vegetables.

Antibiotic	Mode of production	Type of growth	OR	95% CI		*p* value
Ceftazidime	Organic	All	0.957	0.488	1.88	0.5207 (P)
	Conventional		1.045	0.533	2.05	0.5207
	All	In the soil	0.744	0.276	2.01	0.3761(P)
		On the soil	1.189	0.555	2.55	0.4066
		Above the soil	1.020	0.373	2.79	0.5679
	Organic	In the soil	0.452	0.058	3.53	0.3813(P)
		On the soil	1.214	0.612	2.41	0.3485
		Above the soil	0.453	0.025	8.13	0.6658
	Conventional	In the soil	0.923	0.306	2.78	0.5735(P)
		On the soil	0.957	0.488	1.88	0.5207(P)
		Above the soil	1.344	0.484	3.74	0.3678
Cefepime	Organic	All	0.750	0.067	8.36	0.6494(P)
	Conventional		1.333	0.120	14.86	0.6494
	All	In the soil	0.915	0.045	18.56	0.4445
		On the soil	2.281	0.113	46.00	0.5685
		Above the soil	1.207	0.059	24.57	0.3659
	Organic	In the soil	3.222	0.154	67.25	0.1657
		On the soil	1.025	0.092	11.44	0.6979
		Above the soil	7.095	0.324	155.30	0.0829
	Conventional	In the soil	1.422	0.070	29.02	0.3238
		On the soil	3.041	0.273	33.90	0.3506

		Above the soil	1.528	0.075	31.22	0.3064
Imipenem	Organic	All	1.003	0.165	6.09	0.6636
	Conventional		0.997	0.164	6.06	0.6636(P)
	All	In the soil	0.545	0.029	10.13	0.6043
		On the soil	3.836	0.207	70.96	0.3435
		Above the soil	0.719	0.039	13.43	0.5125
	Organic	In the soil	1.920	0.100	36.79	0.2485
		On the soil	1.374	0.226	8.35	0.5294
		Above the soil	4.229	0.210	85.10	0.1275
	Conventional	In the soil	0.847	0.045	15.86	0.4605
		On the soil	2.288	0.377	13.89	0.3152
		Above the soil	0.943	0.050	17.69	0.4275
Ciprofloxacin	Organic	All	9.250	0.459	186.30	0.1150
	Conventional		0.108	0.005	2.18	0.2487(P)
	All	In the soil	0.915	0.045	18.56	0.4445
		On the soil	2.281	0.113	46.00	0.5685
		Above the soil	1.207	0.059	24.57	0.3659
	Organic	In the soil	0.108	0.005	2.18	0.2487(P)
		On the soil	12.670	0.629	255.40	0.0657
		Above the soil	7.095	0.324	155.30	0.0829
	Conventional	In the soil	1.422	0.070	29.02	0.3238
		On the soil	0.247	0.012	4.97	0.5569(P)
		Above the soil	1.528	0.075	31.22	0.3064
Gentamicin	Organic	All	0.855	0.245	2.98	0.5346(P)
	Conventional		1.170	0.335	4.08	0.5346
	All	In the soil	0.546	0.068	4.37	0.4790(P)
		On the soil	3.879	0.489	30.77	0.1495
		Above the soil	0.319	0.018	5.55	0.5818(P)
	Organic	In the soil	0.855	0.048	15.24	0.4531
		On the soil	1.178	0.337	4.12	0.5132
		Above the soil	1.883	0.100	35.32	0.2503
	Conventional	In the soil	0.858	0.106	6.93	0.6805(P)
		On the soil	1.846	0.551	6.19	0.2414
		Above the soil	0.405	0.023	7.06	0.6829(P)
Multidrug resistance	Organic	All	1.517	0.301	7.64	0.4543
	Conventional		0.659	0.131	3.32	0.4543(P)
	All	In the soil	0.452	0.025	8.23	0.6663
		On the soil	4.624	0.256	83.68	0.2628
		Above the soil	1.207	0.059	24.57	0.3659
	Organic	In the soil	4.783	0.234	97.75	0.1149
		On the soil	2.082	0.413	10.50	0.3078

	Above the soil	3.512	0.178	69.18	0.1491
Conventional	In the soil	0.703	0.038	12.88	0.5183
	On the soil	1.517	0.301	7.64	0.4543
	Above the soil	0.756	0.041	13.86	0.4950

[a] (P) indicates an OR value for a protective or negative association; otherwise, values should be interpreted as a positive association.

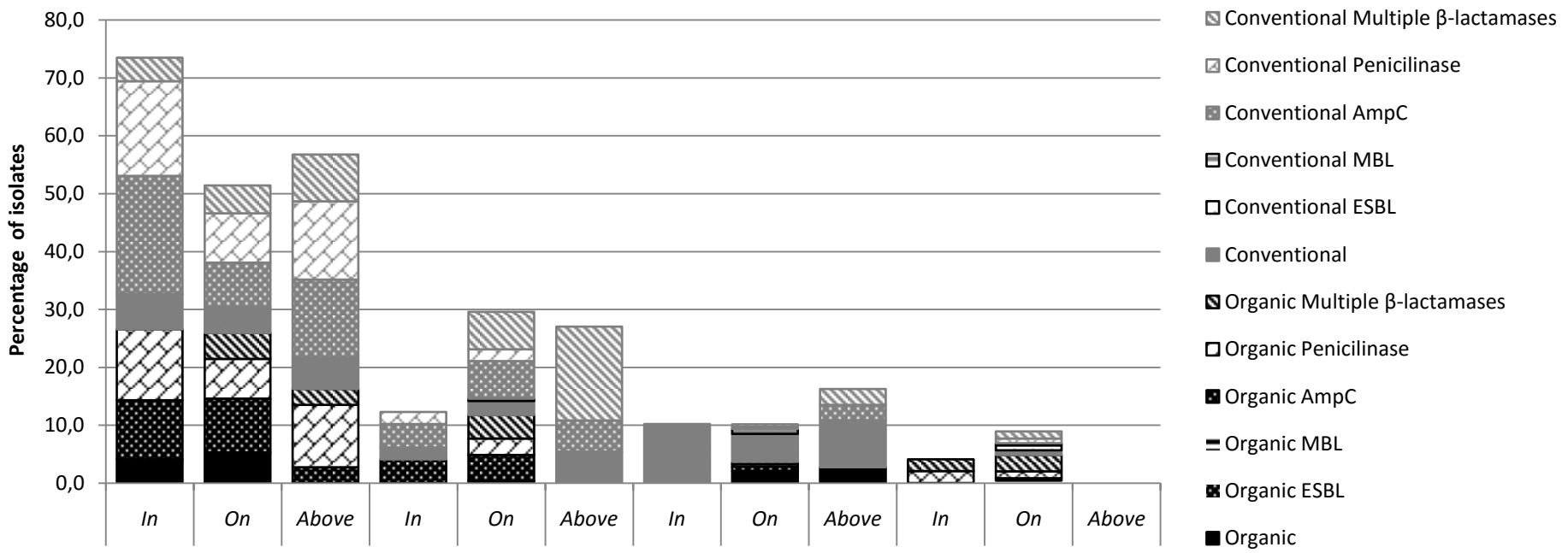

Figure S8.1. Percentage of Gram negative bacteria according with the mode of production and level of growth of fresh produce, taxonomic group and phenotype indicative of penicillinases, AmpC β-lactamase, extended-spectrum β-lactamase (ESBL) and metallo-β-lactamase (MBL) production (n=333).

Table S8.2. Genome analysis of Gram negative isolates carrying integrons.

Isolates	Genome size (bp)	Number of contigs	Average coverage	N50 (bp)	Maximum contig (bp)	Minimum contig (bp)	Protein-coding genes	RNAs
INSali2	4,793,038	42	145.7	344,398	466,839	597	4,471	76
INSali10	4,949,513	63	113.2	251,933	681,923	323	4,618	80
INSali25	5,249,548	165	140.9	99,794	250,299	403	5,254	82
INSali38	5,057,715	107	149.2	128,829	535,543	347	4,904	82
INSali92	4,905,451	87	174.9	167,610	450,780	401	4,686	77
INSali127	6,011,051	81	107.7	185,876	476,313	407	5,731	77
INSali133	6,011,856	77	110.4	156,558	496,480	512	5,729	79
INSali207	3,806,004	79	151.2	213,305	315,226	422	3,688	78
INSali370	4,743,192	110	132.0	131,062	407,142	403	4,559	68
INSali382	6,490,961	246	51.3	53,313	230,406	373	5,938	66
INSali390	5,566,516	88	130.1	177,301	429,033	417	5,240	82

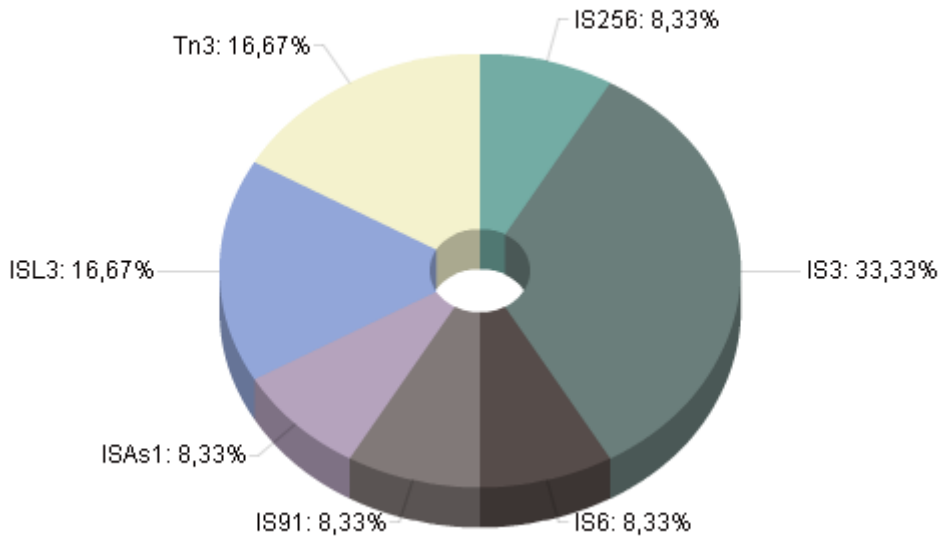

Figure S10.1 Percentage and distribution of insertion sequences detected within *M. morganii* INSRALV892a genome.

Table S12.1. UniProt annotation, quantitative and statistical result on the identified proteins. Available at https://drive.google.com/open?id=0B-gP4W4q5KijZGUzdjJQSm5oeWc.

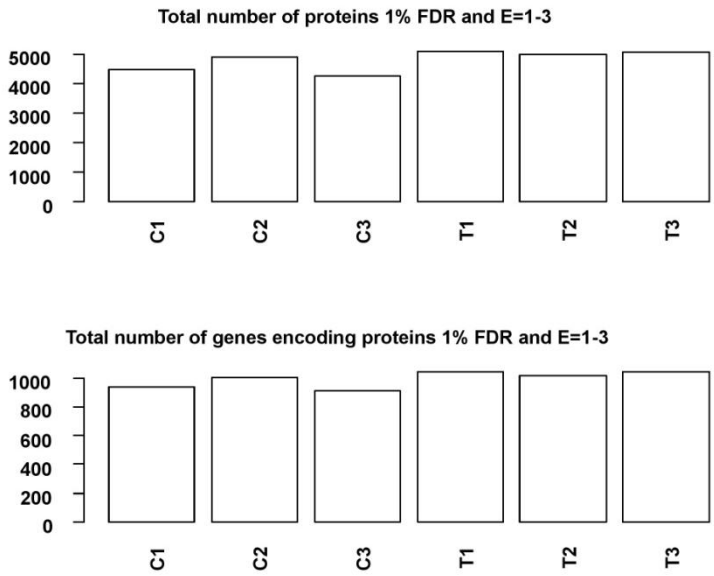

Figure S12.1. Number of proteins and genes encoding proteins identified at 1% FDR across samples. C1, C2, C3: replicas of control EcAmb278; T1, T2, T3: replicas of tetracycline treated EcAmb278.

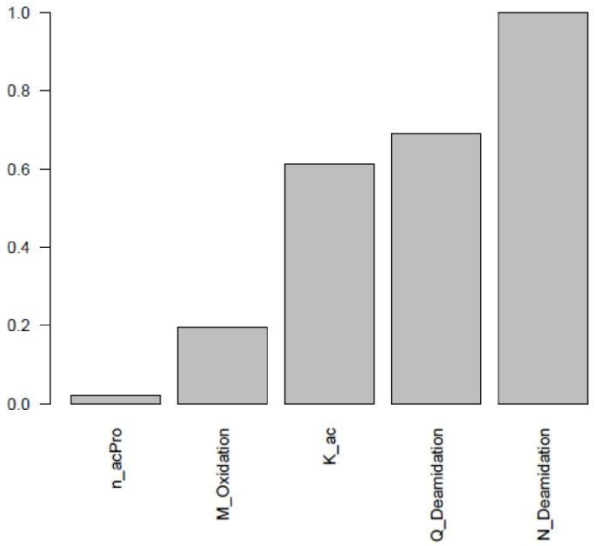

Figure S12.2. Relative frequency of detected protein modifications. N_acPro, N-terminal modification of proteins; M_oxidation, methionine oxidation; K_ac, acetylation of lysine; Q_Deamidation, Glutamine Deamidation; N_Deamidation, Asparagine Deamidation.

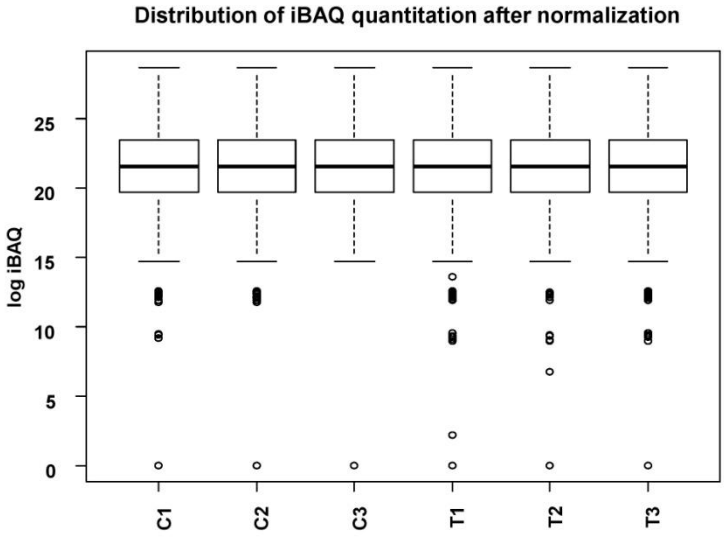

Figure S12.3. Distribution of iBAQ values across samples. C1, C2, C3: replicas of control EcAmb278; T1, T2, T3: replicas of tetracycline treated EcAmb278.

Figure S12.4. Annotated raw spectra assigned to peptides and m/z versus delta m/z plots. Available at https://drive.google.com/open?id=0B-gP4W4q5KijLXpJTlV2SGxySTg

Figure S12.5. Heatmaps summarizing the extracted ion counts for proteins found to be significant regulated by spectral counting. Available at https://drive.google.com/open?id=0B-gP4W4q5KijQnJ6OVA3cjVZLWM.

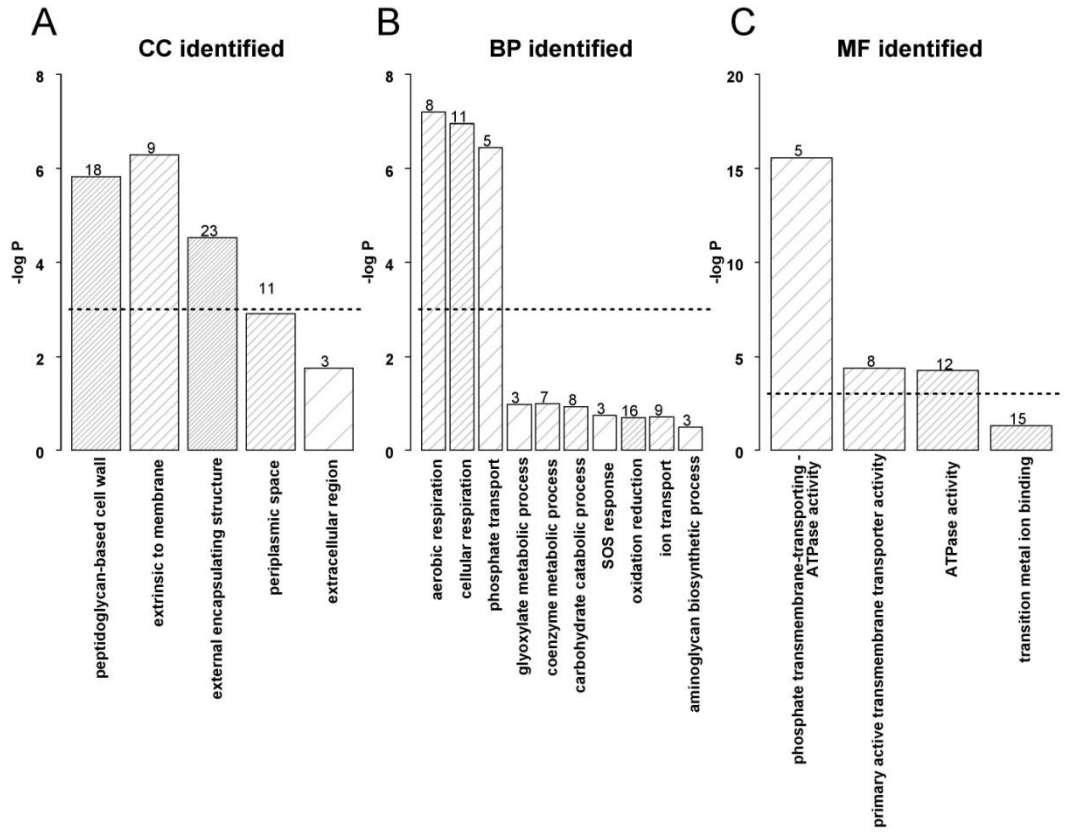

Figure S12.6. Functional enrichment analysis of identified proteins unique for *E. coli* EcAmb278 cultured in the absence of tetracycline. The proteins are analyzed according to cellular component (CC), biological process (BP) and molecular function (MF). The probability of a protein being enriched in a specific subcategory is represented by –log *p*, *p* corresponding to the *p* value. The values on top of each bar correspond to the number of genes enclosed in the specific subcategory which correlates with the pattern fill.

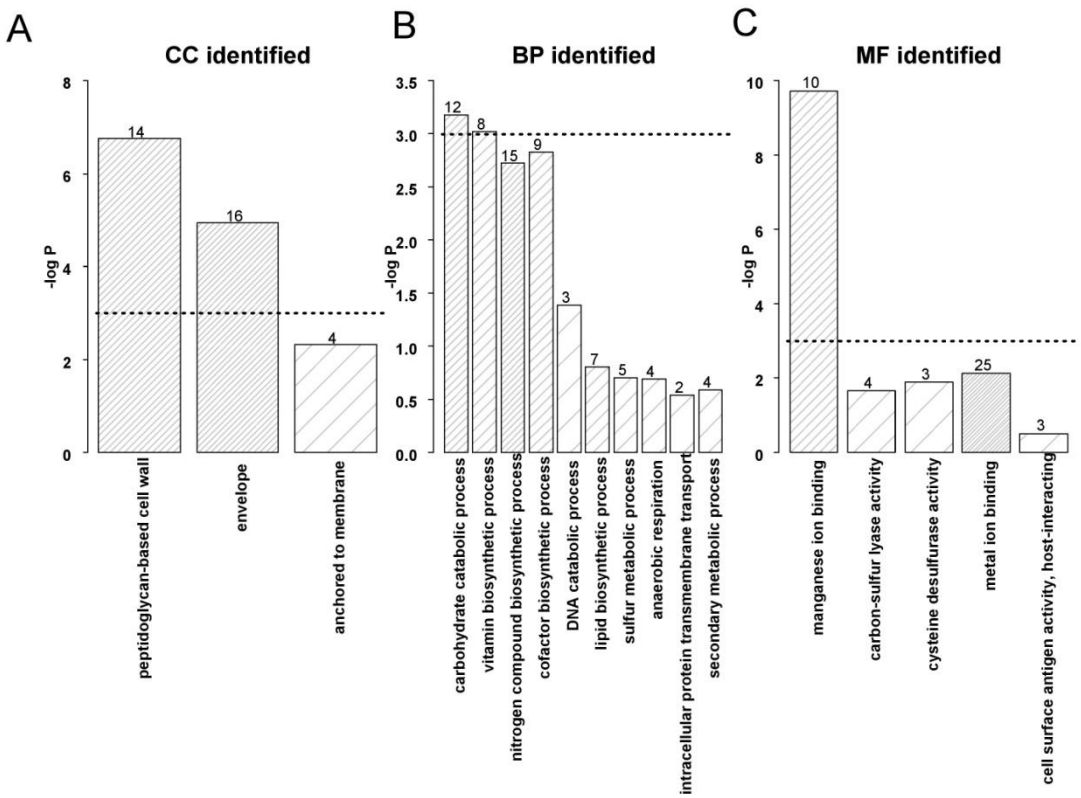

Figure S12.7. Functional enrichment analysis of identified proteins unique for *E. coli* EcAmb278 cultured in the presence of tetracycline. The proteins are analyzed according to cellular component (CC), biological process (BP) and molecular function (MF). The probability of a protein being enriched in a specific subcategory is represented by –log *p*, *p* corresponding to the *p* value. The values on top of each bar correspond to the number of genes enclosed in the specific subcategory which correlates with the pattern fill.

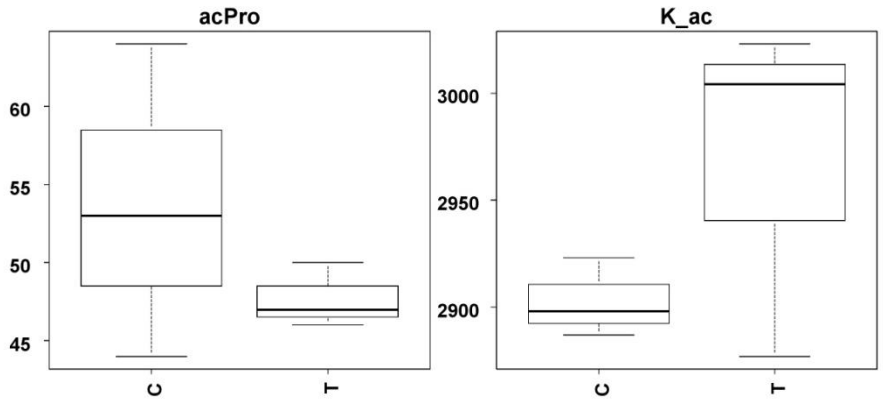

Figure S12.8. Comparison of protein modifications between tetracycline treated and non-treated replicas. acPro, N-terminal modification of proteins; K_ac, acetylation of lysine.